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54 **Macrolide compounds.**

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 EP-A- 0 214 731
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 US-A- 4 321 329

JOURNAL OF THE CHEMICAL SOCIETY, De-
cember 1964, London, GB; A.J. BIRCH et
al.: "Studies in relation to biosynthesis. Part
XXXV. Macrolide antibiotics. PartXII.
Methymycin", pages 5274-5278

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CHEMICAL ABSTRACTS, vol. 90, no. 19, 7th
May 1979, pages 591, column 2 - page 592,
column 1, abstract no. 151957b, Columbus,
Ohio, US; J. INANAGA et al.: "Synthesis of
methynolide", & TENNEN YUKI KAGOBUTSU
TORONKAIKOEN YOSHISHU, 21st 1978,
324-30

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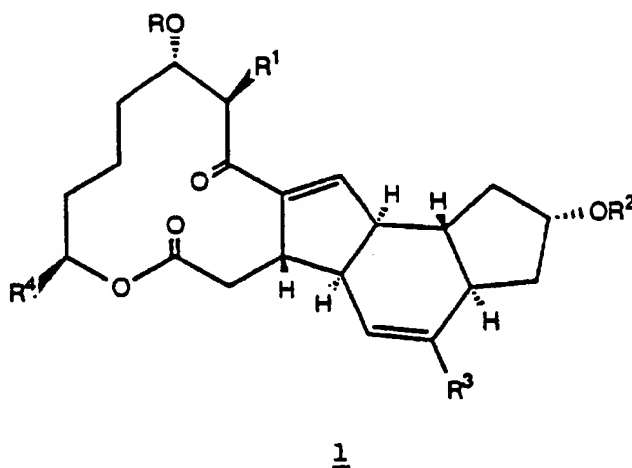
Description

This invention relates to new group of macrolide compounds that have insecticidal and miticidal activity. There is a serious need for new insecticides and miticides because target organisms are rapidly developing resistance to insecticides and miticides in current use. Resistance to insecticides in arthropods is widespread, with at least 400 species being resistant to one or more insecticides. Development of resistance to older insecticides, such as DDT, the carbamates, and the organophosphates, is well known. Resistance has even developed, however, to some of the newer pyrethroid insecticides and miticides. A need exists, therefore, for new insecticides and miticides.

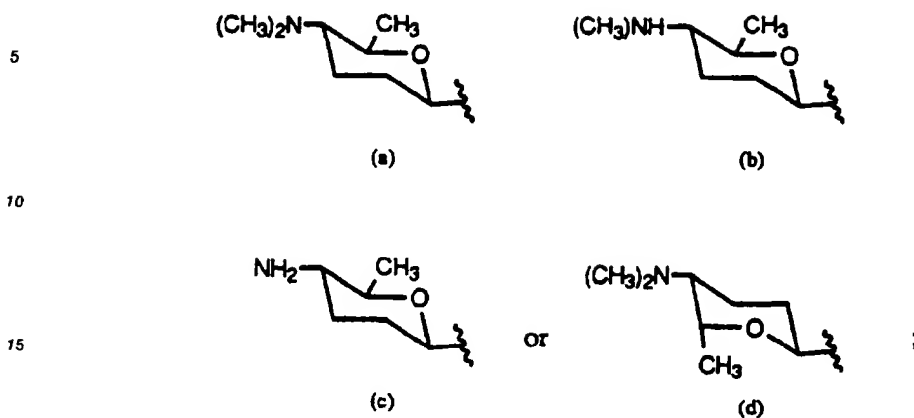
The control of ectoparasites, such as fleas, ticks, biting flies and the like, has long been recognized as an important problem in animal husbandry. The traditional treatments for domestic animals were topically applied insecticides, such as the famous dips for sheep. Indeed, such treatments are still in wide use. Currently, however, the thrust of research has been to find compounds which can be administered to the animals, especially orally, and which will control ectoparasites by poisoning the parasite when it ingests the blood of the treated animal.

This invention relates to a new fermentation product designated "A83543", which is comprised of individual components A83543A, A83543B, A83543C, A83543D, A83543E, A83543F, A83543G, A83543H and A83543J. A83543 and the individual A83543 components are useful for the control of insects, particularly Lepidoptera species, such as Southern armyworm, and Diptera species, such as blow fly, stable fly and mosquito, thus, providing useful insecticidal compositions and methods for reducing population of insects or mites using A83543 compounds.

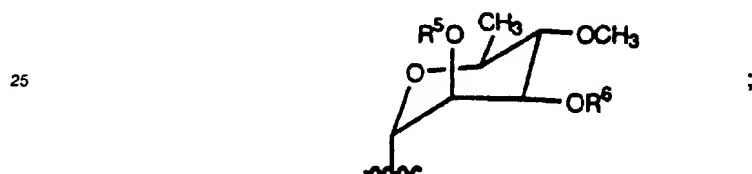
The A83543 compounds of this invention are compounds of formula 1.



wherein R is H or a group selected from



20 R² is



R¹, R³, R⁵ and R⁶ are hydrogen or methyl; and
R₄ is methyl or ethyl;

or an acid addition salt thereof the compounds when R is other than hydrogen.

The preferred compounds of the invention, from the point of view of ease of preparation, are those
35 wherein R, R¹, R³, R⁴, R⁵ and R⁶ are present in one of the following combinations.

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R	R ₁	R ³	R ⁴	R ⁵	R ⁶
(a)	Me	H	Et	Me	Me
(b)	Me	H	Et	Me	Me
(c)	Me	H	Et	Me	Me
(a)	Me	Me	Et	Me	Me
(a)	Me	H	Me	Me	Me
(a)	H	H	Et	Me	Me
45 (d)	Me	H	Et	Me	Me
(a)	Me	H	Et	H	Me
(a)	Me	H	Et	Me	H
H	Me	H	Et	Me	Me
H	Me	Me	Et	Me	Me
50 H	Me	H	Me	Me	Me
H	H	H	Et	Me	Me
H	Me	H	Et	H	Me
H	Me	H	Et	Me	H

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or an acid addition salt thereof the compounds when R is other than hydrogen.

The aminosugar in A83543A has been shown to be β-D-forosamine; and the neutral sugar in A83543A is α-2,3,4-tri-O-methylrhannose.

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Nine A83543 components have been characterized. These components are the formula 1 compounds wherein R is other than H. They have the following structures:

Component	R	R ¹	R ³	R ⁴	R ⁵	R ⁶
A	(a)	Me	H	Et	Me	Me
B	(b)	Me	H	Et	Me	Me
C	(c)	Me	H	Et	Me	Me
D	(a)	Me	Me	Et	Me	Me
E	(a)	Me	H	Me	Me	Me
F	(a)	H	H	Et	Me	Me
G	(d)	Me	H	Et	Me	Me
H	(a)	Me	H	Et	H	Me
J	(a)	Me	H	Et	Me	H

The amino sugar can be removed from the A83543 components to give pseudoaglycones (compounds of formula 1 where R = H). Components A, B, C and G have a common pseudoaglycone (the A83543A pseudoaglycone or pseudo-A). A83543A pseudoaglycone was later found to be produced naturally as an A83543 component. Components D, E, F, H and J each have a unique pseudoaglycone. The A83543 pseudoaglycones have the following structures:

Pseudoaglycone ^a	R ¹	R ³	R ⁴	R ⁵	R ⁶
A83543A	Me	H	Et	Me	Me
A83543D	Me	Me	Et	Me	Me
A83543E	Me	H	Me	Me	Me
A83543F	H	H	Et	Me	Me
A83543H	Me	H	Et	H	Me
A83543J	Me	H	Et	Me	H

^aR = H

The pseudoaglycones are useful as intermediates, for example, to the A83543 components.

The following paragraphs summarize the physical and spectral properties of the A83543 components and pseudoaglycones. In the discussions *infra*, the following abbreviations are used:

EI-MS: electron-impact mass spectrometry
 FAB-MS: fast-atom-bombardment mass spectrometry
 FD-MS: field-desorption mass spectrometry
 HPLC: high performance-liquid chromatography
 IR: infrared
 NMR: nuclear magnetic resonance
 UV: ultraviolet

Characteristics of A83543A

Molecular Weight 731
 Empirical Formula: C₄₁H₆₅NO₁₀
 FD-MS: see Fig. 4
 FAB-MS(M + 1): Found: 732.4706; Calcd. C₄₁H₆₆NO₁₀ = 732.4687 (see fig. 8)
 EI-MS: Found: 731.4612; Calcd. 731.4608 (see fig. 10)
 UV (EtOH) λ_{max}: 243 nm (ε 8,920).
 IR (CHCl₃). ν (lactone) 1713; (conjugated ketone) 1657; multiple peaks for C-H vibrations around 2940 and for C-O vibrations around 1060 cm⁻¹ (see fig. 1)

[α]_D²⁹:

-121.8° (c 1.03, CHCl₃) $[\alpha]_D^{36.5}$:+6.8° (c 1.03, CHCl₃)
acetone-d₆.Table I summarizes the ¹H and ¹³C NMR data observed with A83543A (inTable I: ¹H and ¹³C NMR Data of A83543A in Acetone-d₆.

Position	¹³ C	¹ H ^a
1	172.02	--
2	33.83	3.07/2.45
3	48.13	2.94
4	41.65	3.48
5	129.12	5.86
6	129.66	5.89
7	41.46	2.15
8	36.50	1.99/1.34
9	76.31 ⁺	4.31
10	37.65	2.36/1.36
11	46.42	0.93
12	49.74	2.87
13	147.78	7.01
14	144.27	--
15	202.46	--
16	47.74	3.30
17	80.41	3.53
18	30.33	1.51
19	21.85	1.78/1.17
20	34.45	1.50
21	76.24 ⁺	4.66
22	28.51	1.48
23	8.97	0.81
24	15.71	1.12
1'	96.34	4.81
2'	77.61	3.51
3'	81.87	3.37
4'	82.43	3.00
5'	68.03	3.48
6'	17.64	1.18

Table I: ^1H and ^{13}C NMR Data of A83543A in Acetone- d_6 .
(continued)

Position	^{13}C	$^1\text{H}^a$
2'-OCH ₃	56.66*	3.37
3'-OCH ₃	58.39*	3.41
4'-OCH ₃	60.12	3.45
1''	103.45	4.45
2''	31.24	1.92/1.37
3''	18.14	1.84/1.52
4''	65.34	2.12
5''	73.35	3.56
6''	18.87	1.21
N(CH ₃) ₂	40.38	2.22

^a Some measurements were taken from $^1\text{H}/^{13}\text{C}$ correlation.
*, *Resonances with the same superscript may be inter-
changed.

Characteristics of A83543B

Molecular Weight: 717
Empirical Formula: C₄₀H₆₃NO₁₀
FAB-MS: (see fig. 9)

Characteristics of A83543C

Molecular Weight: 703
Empirical Formula: C₃₉H₆₁NO₁₀
FD-MS: (see fig. 5)

Characteristics of A83543D

Molecular Weight: 745
Empirical Formula: C₄₂H₆₇NO₁₀
UV (EtOH) λ_{max} : 244 nm (ϵ 9,910)
IR (CHCl₃): ν (lactone) 1708; (conjugated ketone) 1658; multiple peaks for C-H vibrations around 2940
and for C-O vibrations around 1070 cm⁻¹ (see fig. 2)

$$[\alpha]_{\text{D}}^{38.9} :$$

-142.9° (c 1.02, CHCl₃)

$$[\alpha]_{\text{D}}^{36.5} :$$

-29.9° (c 1.02, CHCl₃)
FD-MS. see Fig. 6

Table II summarizes the ^1H and ^{13}C NMR data observed with A83543D (in acetone- d_6).

Table II: ^1H and ^{13}C NMR Data of A83543D in Acetone- d_6 .

Position	^{13}C	$^1\text{H}^a$
1	172.68	--
2	34.38	3.08/2.43
3	49.01	2.90
4	42.83	3.47
5	123.27	5.54
6	137.26	--
6- CH_3	20.81	1.74
7	44.41	2.18
8	35.61	2.01/1.45
9	76.72	4.32
10	38.64	2.37/1.37
11	47.04	1.02
12	50.05	2.78
13	148.47	7.04
14	145.19	--
15	203.16	--
16	48.47	3.30
17	81.03	3.53
18	30.99	1.49
19	22.51	1.78/1.19
20	35.12	1.49
21	76.84	4.65
22	29.16	1.48
23	9.55	0.81
24	16.32	1.12
1'	97.11	4.85
2'	78.33	3.54
3'	82.58	3.40

Table II: ^1H and ^{13}C NMR Data of A83543D in Acetone- d_6 .
(continued)

Position	^{13}C	$^1\text{H}^a$
4'	83.15	3.03
5'	68.71	3.50
6'	18.26	1.18
2'-OCH ₃	57.31*	3.40
3'-OCH ₃	59.02*	3.43
4'-OCH ₃	60.71	3.47
1''	104.14	4.47
2''	31.96	1.94/1.39
3''	18.83	1.81/1.49
4''	66.06	2.12
5''	74.12	3.55
6''	19.42	1.20
N(CH ₃) ₂	40.99	2.21

^aSome assignments taken from $^1\text{H}/^{13}\text{C}$ correlation.

*Resonances may be interchanged.

Characteristics of A83543E

Molecular Weight: 717

Empirical Formula: $\text{C}_{40}\text{H}_{63}\text{NO}_{10}$

FAB-MS ($M+1$): Found: 718.4526; Calcd. $\text{C}_{40}\text{H}_{64}\text{NO}_{10}$ = 718.4530

UV (EtOH) λ_{max} : 244 nm (ϵ 8,600)

IR (KBr) (see fig. 13)

Table III summarizes the ^1H and ^{13}C NMR data observed with A83543E (in acetone- d_6).

Table III: ^1H and ^{13}C NMR Data of A83543E in Acetone- d_6 .

Position	^{13}C	$^1\text{H}^a$
1	172.46	--
2	34.95	3.06/2.40
3	48.88	2.95
4	42.11	3.43
5	129.78	5.86
6	130.39	5.90
7	42.11	2.14
8	37.18	1.96/1.39
9	77.06	4.33
10	38.31	2.36/1.36
11	47.18	0.93
12	50.40	2.86
13	148.37	7.06
14	144.84	--
15	203.09	--
16	48.05	3.34
17	81.35	3.55
18	34.98	1.62/1.48
19	22.25	1.77/1.13
20	33.73	1.50
21	72.97	4.68
22	21.61	1.12
23	--	--
24	16.52	1.13
25	97.11	4.83
26	78.36	3.55
27	82.55	3.37
28	83.13	3.02
29	68.72	3.50
30	18.26	1.18

Table III: ^1H and ^{13}C NMR Data of A83543E in Acetone- d_6 .
(continued)

Position	^{13}C	$^1\text{H}^a$
2'-OCH ₃	59.01	3.43
3'-OCH ₃	57.30	3.40
4'-OCH ₃	60.69	3.46
1''	104.24	4.47
2''	32.00	1.93/1.39
3''	18.86	1.82/1.50
4''	66.06	2.12
5''	74.13	3.57
6''	19.42	1.21
N(CH ₃) ₂	40.99	2.21

^a Some measurements were taken from $^1\text{H}/^{13}\text{C}$ correlation.

Characteristics of A83543F

Molecular Weight: 717

Empirical Formula: C₄₀H₆₃NO₁₀

FAB-MS (M+1): Found: 718.4534; Calcd. C₄₀H₆₄NO₁₀ = 718.4530

UV (EtOH) λ_{max} : 243 nm (ϵ 10,500) and 282 nm (ϵ 109)

IR (KBr): (see fig. 14)

Table IV summarizes the ^1H and ^{13}C NMR data observed with A83543F (in acetone- d_6).

Table IV: ^1H and ^{13}C NMR Data of A83543F in Acetone- d_6 .

Position	^{13}C	$^1\text{H}^a$
1	172.60	--
2	34.50	3.06/2.42
3	48.82	2.95
4	42.46	3.45
5	129.56	5.87
6	130.39	5.92
7	42.19	2.16
8	37.18	1.97/1.35
9	77.15	4.65
10	38.30	2.33/1.34
11	46.89	0.94
12	50.34	2.84
13	148.86	7.03
14	145.73	--
15	198.68	--
16	45.49	3.22/2.50
17	74.17	3.58
18	30.74	1.52
19	22.41	1.70/1.17
20	34.45	1.51
21	77.09	4.32
22	29.05	1.48
23	9.56	0.81
1'	97.19	4.83
2'	78.38	3.53
3'	82.58	3.38
4'	83.15	3.00
5'	68.74	3.48
6'	18.25	1.18

Table IV: ^1H and ^{13}C NMR Data of A83543F in Acetone- d_6 .
(continued)

Position	^{13}C	$^1\text{H}^a$
2'-OCH ₃	59.02	3.43
3'-OCH ₃	57.31	3.40
4'-OCH ₃	60.69	3.47
1''	100.19	4.53
2''	32.41	1.80/1.38
3''	18.86	1.83/1.53
4''	66.16	2.13
5''	74.01	4.01
6''	19.46	1.22
N(CH ₃) ₂	41.01	2.22

^aSome measurements were taken from $^1\text{H}/^{13}\text{C}$ correlation.

Characteristics of A83543G

Molecular Weight: 731

Empirical Formula: C₄₁H₆₅NO₁₀

FAB-MS (M+1): Found: 732.4661; Calcd. C₄₁H₆₅NO₁₀ = 732.4687

UV (EtOH) λ_{max} 243 nm (ϵ 8,970)

IR (KBr): (see fig. 15)

Table V summarizes the ^1H and ^{13}C NMR data observed with A83543G (in acetone- d_6).

Table V: ^1H and ^{13}C NMR Data of A83543G in Acetone- d_6 .

Position	^{13}C	$^1\text{H}^a$
1	172.59	--
2	34.67	3.04/2.46
3	48.72	2.94
4	42.25	3.50
5	129.85	5.84
6	130.26	5.89
7	42.02	2.14
8	37.12	1.95/1.34
9	76.99	4.32
10	38.28	2.36/1.36
11	47.23	0.91
12	50.43	2.87
13	148.28	7.04
14	144.61	--
15	203.20	--
16	47.94	3.30
17	81.73	3.57
18	35.20	1.55
19	21.68	1.64/1.16
20	31.41	1.64/1.36
21	76.47	4.64
22	28.84	1.48
23	9.61	0.80
24	15.29	1.18
1'	96.98	4.81
2'	78.23	3.52
3'	82.46	3.37
4'	83.05	2.29
5'	68.64	3.49
6'	17.18	1.12

Table V: ^1H and ^{13}C NMR Data of A83543G in Acetone- d_6 .
(continued)

Position	^{13}C	$^1\text{H}^a$
2'-OCH ₃	58.97	3.42
3'-OCH ₃	57.25	3.39
4'-OCH ₃	60.70	3.46
1''	99.51	4.80
2''	29.62	1.87/1.48
3''	19.31	1.73
4''	62.13	2.29
5''	69.93	4.20
6''	18.22	1.17
N(CH ₃) ₂	43.47	2.24

^aSome measurements were taken from $^1\text{H}/^{13}\text{C}$ correlation.

Characteristics of A83543H

Molecular Weight: 717
Empirical Formula: C₄₆H₆₃NO₁₀
UV (EtOH) λ_{max} : 243 nm (ϵ 10,100)*
IR (KBr): see fig. 16*

Characteristics of A83543J

Molecular Weight: 717
Empirical Formula: C₄₆H₆₃NO₁₀
UV (EtOH) λ_{max} : 243 nm (ϵ 10,100)*
IR (CHCl₃): see fig. 16*

A83543H and J are separated from A83543 as a mixture (H:J ratio = 58:42) which can be separated by analytical high performance liquid chromatography (HPLC), as described *infra*. The structures assigned to A83543H and J are based on ^1H and ^{13}C NMR studies of the A83543 H:J mixture in acetone- d_6 . The NMR spectra resemble and were compared with those of A83543A. The major changes in the H and J spectra center around the rhamnose sugar. Components H and J have only two OCH₃'s in that sugar. In component H, H-1' is shifted about .1 δ in the ^1H NMR spectrum and 3 δ in the ^{13}C NMR spectrum (4.81 and 99.68, respectively). These shifts are due to the absence of methyl on the methoxy at the 2' position. The shifts in component J correspond to a similar absence of the methyl on the methoxy at the 3' position.

Characteristics of A83543A Pseudoaglycone

Molecular Weight: 590
Empirical Formula: C₃₃H₅₀O₉
UV (EtOH) λ_{max} : 243 nm (ϵ 10,300)
IR (CHCl₃): ν (lactone) 1724; (conjugated ketone) 1652; multiple peaks for C-H vibrations around 3017; multiple peaks for C-O vibrations around 1140 cm⁻¹ (see fig. 3)

* determined on an A83543H:J (58:42) mixture

* determined on an A83543 H:J (58:42) mixture

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FD-MS: see Fig. 7

EI-MS: see Fig. 11

Characteristics of A83543D Pseudoaglycone

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Molecular Weight. 604

Empirical Formula. $C_{34}H_{52}O_3$

The A83543 components can be separated from each other using one of the following analytical HPLC systems:

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SYSTEM I

Column: ODS, 3 μ , 4.5 x 50 mm (IBM)
 Solvent: $CH_3OH:CH_3CN:H_2O$ (2:2:1)
 15 Flow Rate: 1.0 mL/min
 Detection: UV at 245 nm
 Temperature: Room Temperature

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Component	Retention Time (min)
A	8.50
B	6.15
C	3.92
D	11.47

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SYSTEM II

Column. 4.6 x 100 mm, ODS (AQ-301, S-5, YMC, Inc., Mt. Freedom, NJ)
 30 Solvents $CH_3OH:CH_3CN:0.05\% NH_4OAc(H_2O)$
 (A) 35:35:30 - pH 7.8
 (B) 45:45:10 - pH 6.7
 Flow Rate: 2.0 mL/min
 Run time: 35 min
 35 Detection: UV, 250 nm
 Gradient: 10% B to 25% B in 20 min; to 50% B in 30 min

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Component	Retention Time (min)
A	22.62
A-pseudoaglycone	7.27
B	12.65
C	10.62
D	25.47
E	19.22
F	16.30
G	18.92
H	16.30
J	17.50

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SYSTEM III

55 Column 4.6 x 100 mm, ODS (AQ-301, S-5, YMC, Inc., Mt. Freedom, NJ)
 Solvents $CH_3OH:CH_3CN:0.05\% NH_4OAc(H_2O)$ 35:35:30 - pH 6.0
 Flow Rate: 2.0 mL/min
 Run time: 10 min

Detection: UV, 250 nm

Component	Retention Time (min)
F	4.32
H	3.42
J	3.42

The A83543 components are not soluble in water, but are soluble in solvents such as methanol, ethanol, dimethylformamide, dimethyl sulfoxide, acetonitrile, acetone and the like.

A83543 and individual components, such as A83543A, A83543B, A83543C, A83543D, A83543E, A83543F, A83543G, A83543H, and A83543J can react to form various salts. All such forms of these compounds are part of this invention. A83543 salts are useful, for example, for separating and purifying A83543. In addition, some salts have an improved solubility in water.

A83543 salts are prepared using standard procedures for salt preparation. For example, A83543 can be neutralized with an appropriate acid to form an acid addition salt.

The acid addition salts are particularly useful. Representative suitable salts include those salts formed by standard reactions with both organic and inorganic acids such as, for example, sulfuric, hydrochloric, phosphoric, acetic, succinic, citric, lactic, maleic, fumaric, cholic, pantoic, mucic, glutamic, camphoric, glutaric, glycolic, phthalic, tartaric, formic, lauric, stearic, salicylic, methanesulfonic, benzenesulfonic, sorbic, picric, benzoic, cinnamic and like acids.

In discussions of utility, the term "A83543 compound" preferably denotes a member selected from the group consisting of A83543, individual components A83543A, A83543B, A83543C, A83543D, A83543E, A83543F, A83543G, A83543H, and A83543J and their acid addition salts.

Fermentation product A83543 is produced by culturing a strain of the novel microorganism Saccharopolyspora spinosa selected from NRRL 18395, NRRL 18537, NRRL 18538 and NRRL 18539, or an A83543-producing mutant thereof, under submerged aerobic conditions in a suitable culture medium until a recoverable amount of A83543 is produced. As those skilled in fermentation processes will recognize, the ratio of the components in A83543 will vary, depending upon the fermentation conditions used to produce it. In general, A83543 contains about 85-90% A83543A, about 10-15% A83543D and minor amounts of A83543B, C, E, F, G, H and J and A83543A pseudoaglycone. Individual components such as A83543A, A83543B, A83543C, A83543D, A83543E, A83543F, A83543G, A83543H, and A83543J and A83543A pseudoaglycone can be separated and isolated as described *infra*.

Thus, another aspect of this invention is a process for producing an A83543 compound which comprises cultivating a Saccharopolyspora spinosa strain selected from NRRL 18395, NRRL 18537, NRRL 18538 or NRRL 18539, or an A83543-producing mutant thereof, in a culture medium containing assimilable sources of carbon, nitrogen, and inorganic salts under submerged aerobic fermentation conditions until a recoverable amount of A83543 is produced. Individual components can then be isolated by techniques known *per se* in the art.

This invention also relates to a biologically purified culture of the microorganism Saccharopolyspora spinosa selected from NRRL 18395, NRRL 18537, NRRL 18538 or NRRL 18539, or an A83543-producing mutant thereof. These microorganisms are useful because they produce A83543.

For convenience in the discussions which follow, the strains have been given the following designations: A83543.1, A83543.3, A83543.4 and A83543.5. Culture A83543.1 was obtained by chemical mutation of a culture (A83543) isolated from a soil sample collected from the Virgin Islands. Cultures A83543.3, A83543.4 and A83543.5 were obtained from derivatives of the A83543.1 culture by chemical mutations.

Cultures A83543.1, A83543.3, A83543.4 and A83543.5 have been deposited and made part of the stock culture collection of the Midwest Area Northern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, 1815 North University Street, Peoria, Illinois, 61604, from which they are available to the public under the following accession numbers:

NRRL No.	Strain No.
18395	A83543.1
18537	A83543.3
18538	A83543.4
18539	A83543.5

Taxonomic studies of culture A83543.1 were carried out by Frederick P. Mertz of the Lilly Research Laboratories. Based on these studies, the microorganisms A83543.1, A83543.3, A83543.4 and A83543.5 are classified as members of a new species of the genus Saccharopolyspora, which is called Saccharopolyspora spinosa sp. nov. This classification is based on direct laboratory comparisons and examination of published descriptions of similar species.

Methods Used

The methods followed were those recommended by the International Streptomyces Project (ISP) for the characterization of Streptomyces species [E. B. Shirling and D. Gottlieb, "Methods for Characterization of Streptomyces Species," Int. J. Syst. Bacteriol. 16:313-340 (1966)] and those recommended for the characterization of Nocardia species by R. E. Gordon, D. A. Barnett, J. E. Handershan and C. H. Pang, "Nocardia coeliaca, Nocardia autotrophica, and the Nocardin Strain", Int. J. Syst. Bacteriol. 24(1), 54-63 (1974).

ISCC-NBS Centroid Color Charts, standard sample No. 2106 (National Bureau of Standards, 1958, U.S. Department of Commerce, Washington, D.C.) were used to assign color names to the reverse side and to aerial hyphae.

Morphology was studied using an optical light microscope and a scanning electron microscope (SEM). The isomer of diaminopimelic acid (DAP) and the carbohydrates in hydrolysates of whole cells were established by the chromatographic methods of Becker et al. [B. Becker, M. P. Lechevalier, R. E. Gordon and H. E. Lechevalier, "Rapid Differentiation between Nocardia and Streptomyces by Paper Chromatography of Whole-cell Hydrolysates," Appl. Microbiol. 12, 421-423 (1964)] and of Lechevalier and Lechevalier [M. P. Lechevalier and H. Lechevalier, "Chemical Composition as a Criterion in the Classification of Aerobic Actinomycetes," Int. J. Syst. Bacteriol. 20, 435-443 (1970)].

Phospholipids were determined by the procedure of M. P. Lechevalier and H. Lechevalier [in A University Laboratory Approach, Dietz and Thayer (eds.), Society for Industrial Microbiology Special Publication No. 6, Arlington, VA, pp. 227-233 (1980)].

Menaquinone composition was determined by following the procedures of R. M. Kroppenstedt [in Chemical Methods in Bacterial Systematics, M. Goodfellow and D. E. Minnikin (eds.), 1985, pp. 173-196] and M. D. Collins (ibid., pp. 267-285).

Resistance to antibiotics was measured by padding antibiotic sensitivity discs onto the surface of seeded ISP No. 2 agar plates.

Starch hydrolysis was determined by testing for the presence of starch with iodine on ISP No. 4 (inorganic salts-starch) agar plates.

Fatty acid analysis was done using the HP 5898A Microbial Identification System [see L. Miller and T. Berger, "Bacterial Identification by Gas Chromatography of Whole Cell Fatty Acids," Hewlett-Packard Application Note 228-41, 8pp (1985)].

Fatty acid methyl esters were made from lyophilized whole cells grown under identical conditions. Principal component analysis was two dimensional and computer generated Units of measurement in the principal component plot (shown in Figure 12) are standard deviations.

Mycolic acids were determined by the methods proposed by Minnikin [D. E. Minnikin, I. G. Hutchinson and A. B. Caldicott, "Thin-Layer Chromatography of Methanolysates of Mycolic Acid-Containing Bacteria," J. Chromatography 188, 221-233 (1980)].

Cultural Characteristics

Culture A83543.1 grew well on both complex and defined media. The culture produced aerial mycelia on all the media used. The aerial spore-mass color was predominantly light yellowish-pink, but was white on a number of the media.

The reverse side was yellow to yellow-brown. No distinctive pigmentation was present. A soluble brown pigment was released into the medium in some media.

The cultural characteristics are summarized in Table VI

Table VI: Cultural Characteristics of A83543.1^a

Medium	Growth	Reverse Color	Aerial Mycelium		Soluble Pigment
			Growth	Color	
ISP medium 2	Abundant	76. l. yBr.	Abundant	92. yWhite	None
ISP medium 3	Good	264. l. Gray	Good	263. White	None
ISP medium 4	Good	92. yWhite	Good	263. White	None
ISP medium 5	Abundant	73. p. OY	Abundant	31. p. yPink	l. Brown
ISP medium 7	Abundant	77. m. yBr	Abundant	31. p. yPink	l. Brown
AIA Agar ^b	Abundant	89. p. Y	Abundant	31. p. yPink	Brown
ATCC No. 172	Abundant	92. yWhite	Abundant	31. p. yPink	None
Bennetts	Abundant	92. yWhite	Abundant	31. p. yPink	l. Brown
Calcium-malate	Good	73. p. OY	Fair	9. pk. White	Brown
Chitin	Fair	92. yWhite	Fair	263. White	None
Czapek	Good	92. yWhite	Good	263. White	None
Emerson	Abundant	76. l. yBr	Abundant	31. p. yPink	Brown
Glucose-Asparagine	Good	90. gy. Y	Fair	31. p. yPink	l. Brown

Table VI: Cultural Characteristics of A83543.1^a (cont.)

Medium	Growth	Reverse Color	Aerial Mycelium Growth	Color	Soluble Pigment
Glycerol- Glycine	Abundant	78. d. yBr	Abundant	80. gy. yBr	d.Brown
Nutrient	Abundant	90. gy. Y	Abundant	31. p. yPink	l.Brown
TPO ^c	Abundant	90. gy. Y	Abundant	31. p. yPink	None
TWA ^d	Fair	93. y Gray	Poor	263. White	None
YDA ^e	Abundant	77. m. yBr	Fair	9. pk. White	d.Brown

^a Incubated at 30°C for 21 days.^b Actinomycete Isolation Agar, Difco.^c Tomato paste oatmeal agar. In The Actinomycetes, vol. 2, S. A. Waksman, The Williams and Wilkins Co., Baltimore, 1961).^d Tap water agar (Gordon, Barnett, Handerhan and Pang, supra).^e Yeast dextrose agar (Gordon, Barnett, Handerhan and Pang, supra).55 Morphological Characteristics

Culture A83543.1 produced an extensive substrate mycelium that fragmented in liquid fermentation. No fragmentation was observed when the culture was grown on agar media.

White circular colonies, 8-10 mm in diameter with raised center and yellow-brown reverse color, were observed when the culture was plated on ISP medium 1.

Well-formed aerial hyphae were present on most of the media. The aerial hyphae were segmented into long chains of spores arranged as hooks and open loops. Spirals were also observed, but they were short and incomplete.

The general morphology was Rectus-flexibilis (RA).

Aerial hyphae had a distinctive bead-like appearance, with many empty spaces in the spore chain. This feature demonstrated that a spore sheath encased the spore chain. This spore sheath was covered with very distinctive spines. The spines were approximately 1 μm long and were rounded on the end.

The spore shape was oblong and averaged approximately 1.1 x 1.5 μm in size. The spore-chain length was well over 50 spores. No zig-zag characteristics, sclerotia, sporangia or motile cells were observed.

Physiological Characteristics

Culture A83543.1 produced acid from the following carbohydrates: adonitol, D-arabinose, erythritol, fructose, glucose, glycerol, mannitol, mannose, ribose and trehalose.

The culture did not produce acid from: L-arabinose, cellobiose, cellulose, dextrin, dulcitol, ethanol, galactose, glycogen, inositol, inulin, lactose, maltose, melizitose, melebiose, α -methyl-D-glucoside, raffinose, L-rhamnose, salicin, sorbitol, L-sorbose, sucrose, xylitol or xylose

Growth was observed with galactose, maltose and melizitose, but no acid was produced from these carbohydrates.

Culture A83543.1 used the following organic acids as sodium salts: acetate, butyrate, citrate, formate, lactate, malate, propionate, pyruvate and succinate. The culture did not use benzoate, mucate, oxalate or tartrate.

A83543.1 decomposed allantoin, calcium malate, casein, elastin, hippurate, hypoxanthine, testosterone, L-tyrosine and urea. It was unable to decompose adenine, esculin, guanine, starch or xanthine.

A83543.1 produced catalase, phosphatase, urease and H_2S . It liquefied gelatin and reduced nitrate. It was not resistant to lysozyme and did not produce melanoid pigments. It neither peptonized nor hydrolyzed skim milk. A83543.1 tolerated levels of NaCl up to and including 11%. It was unable to survive 50 °C for 8 hours, but grew at temperatures between 15 ° and 37 °C

A83543.1 was resistant to cephalothin (30 μg), penicillin G (10 units) and rifampin (5 μg). It was sensitive to bacitracin (10 units), gentamicin (10 μg), lincomycin (2 μg), neomycin (30 μg), oleandomycin (15 μg), streptomycin (10 μg), tetracycline (30 μg), tobramycin (10 μg) and vancomycin (30 μg).

Cell-Wall Analysis

Hydrolyzed whole cells of A83543.1 contained meso-diaminopimelic acid. Diagnostic sugars in the whole-cell extracts were galactose and arabinose. Thus, A83543.1 has a Type IV cell-wall pattern and a Type A sugar pattern (Lechevalier and Lechevalier, *supra*). The cells do not contain mycolic acids.

Phospholipid determinations on the whole cells indicated the presence of phosphatidyl choline and cardiolipin. No phosphatidyl ethanolamine was detected. Thus, A83543.1 has a Type PIII phospholipid pattern [M. P. Lechevalier, A. E. Stern and H. A. Lechevalier, "Phospholipids in the Taxonomy of Actinomycetes," in *Actinomycetes*, Zbl. Bakt. Suppl. 11, K. P. Schaal and G. Pulverer (eds), Gustav Fischer Verlag, New York, 1981].

The major menaquinone detected was MK-9(H_4). A minor amount of MK-9(H_6) was observed.

Phage Plating

A number of Streptomyces, Saccharopolyspora and Amycolatopsis phages were plated on A83543.1. No plaques were observed.

Identity of A83543.1

As discussed, *supra*, culture A83543.1 has a type IV cell-wall pattern and a type A whole-cell sugar pattern. The following thirteen genera have this pattern of cell chemistry: Nocardia, Rhodococcus, Corynebacterium, Caseobacter, Mycobacterium, Faenia (Micropolyspora), Pseudonocardia, Saccharomonospora, Saccharopolyspora, Actinopolyspora, Amycolata, Amycolatopsis and Kibdelosporangium. These genera are distinguished by the presence or absence of mycolic acids, by fatty acid composition,

and by phospholipid and menaquinone types. Faenia, Pseudonocardia and Saccharopolyspora have chemotaxonomic characteristics identical to those of A83543.1, but these genera differ from A83543.1 in morphological and cultural properties.

The genus Faenia (Micropolyspora) has smooth spores and short spore chains which are borne on both aerial and substrate hyphae. Its aerial hyphae are sparse and white in color. It is a thermophile which grows at 60 °C. A83543.1 has none of these properties and thus differs from Faenia.

The genus Pseudonocardia has spores on both aerial and substrate hyphae. It is distinguished by acropetal budding and blastospores. It has a characteristic zig-zag morphology of the hyphae. The hyphae have been described as articulated but non-septate [see A. Henssen and D. Schafer, "Amended Description of the Genus Pseudonocardia Henssen and Description of a New Species Pseudonocardia spinosa Schafer," Int. J. Syst. Bacteriol. 21:29-34 (1971)]. It grows very slowly. Fragmentation is absent or rarely observed. A83543.1 has none of these properties.

The genus Saccharopolyspora is characterized by a spore sheath and a distinctive bead-like appearance of the spore chain. This feature is very prominent in A83543.1. Fragmentation has also been observed with the genus Saccharopolyspora. The type species, S. hirsuta, was isolated from sugar-cane bagasse. The parent culture from which A83543.1 was obtained was isolated from a sugar mill. Since A83543.1 has many properties of this genus, it is, therefore, considered to be a strain of Saccharopolyspora.

The only validly published species in the genus Saccharopolyspora are S. erythraea and S. hirsuta. Known subspecies are S. hirsuta subsp. taberi and S. hirsuta subsp. kobensis. A83543.1 differs from these strains in either aerial and reverse color or in production of soluble pigments.

Biochemical similarity was measured by constructing a table of similarity coefficients based on as many biochemical measurements as possible. The coefficient of Jaccard S_j and the simple matching coefficient S_{sm} were used [see W. Kurylowicz, A. Paszkiewicz, W. Woznicka, W. Kurzatkowski and T. Szulga, "Numerical Taxonomy of Streptomycetes," Polish Medical Publishers, Warsaw, 1975, p. 37]

Table VII summarizes these similarity coefficients.

Table VII

Similarity Coefficients For A83543.1 and <u>Saccharopolyspora</u> species		
Culture	S_{sm}	S_j
A83543.1	100	100
<u>S. hirsuta</u> subsp. <u>taberi</u>	68	57
<u>S. hirsuta</u> subsp. <u>kobensis</u>	67	60
<u>S. erythraea</u>	63	54
<u>S. hirsuta</u>	55	50

Fatty acid analysis of A83543.1 and the known Saccharopolyspora species showed that each has both saturated and branched-chain fatty acids. The fatty acid composition of A83543.1 is similar, but not identical, to that of the other species. Table VIII compares the fatty acid compositions of A83543.1 and the known Saccharopolyspora species.

Table VIII: Percentage Fatty Acid Composition of A83543.1 and *Saccharopolyspora* Strains

Fatty Acid	A83543.1	<i>S. erythraea</i>	<i>S. hirsuta</i>	<i>S. hirsuta</i> subsp. <i>kobensis</i>
15:0 Iso	11.80	17.86	15.44	17.94
15:0 Anteiso	0.64	1.33	1.38	1.25
16:0 Iso	22.47	25.70	15.61	19.05
16:1 Trans 9	1.15	--	4.14	2.63
17:1 Iso F	7.22	7.97	--	--
17:1 Iso G	--	--	3.75	6.82
17:0 Iso	17.59	13.37	26.26	19.41
17:0 Anteiso	15.30	12.46	14.19	12.72
17:1 B	4.77	1.90	--	0.67
17:1 C	1.65	--	2.70	1.81
17:0	2.74	1.01	1.43	0.94
16:1 2OH	1.27	2.07	4.74	4.85
18:1 Iso F	7.57	11.31	6.83	7.47
TBSA 10Me ^a	1.15	1.34	1.77	1.00
18:0				

^aTBSA = tuberculostearic acid

Principal component analysis of the fatty acid compositions shown in Table VIII show sufficient scattering to suggest the cultures are all distinct species within the same genus. The principal component plot of the Table VIII data is presented in Figure 12.

Table IX compares the physiological characteristics of A83543.1 to those of the existing *Saccharopolyspora* species and subspecies.

Table IX: Differential Physiological Characteristics of A83543.1 and Saccharopolyspora Species

Characteristic	A83543.1	<u>S. erythraea</u>	<u>S. hirsuta</u>	<u>S. hirsuta</u> subsp. taberi	<u>S. hirsuta</u> subsp. kobensis
Decomposition of:					
adenine	-	+	+	+	+
esculin	-	+	+	+	+
starch	-	+	+	+	+
xanthine	-	+	+	+	+
allantoin	+	-	-	-	ND
Nitrate reduction	+	+	+	+	+
Phosphatase	+	-	+	-	ND
Utilization of:					
benzoate	-	-	+	-	ND
mucate	-	-	+	-	ND
oxalate	-	-	+	-	ND
Temperature range (°C)	15-37	20-42	25-50	20-42	20-42
NaCl tolerance (%)	11	ND*	15	ND	12
Acid produced from:					
arabinose	+	+	-	+	-
cellobiose	-	+	+	+	+
dextrin	-	+	+	+	ND
galactose	-	+	+	+	+
inositol	-	+	+	+	-
maltose	-	+	+	+	+
melezitose	-	+	-	+	ND

Table IX: Differential Physiological Characteristics of A83543.1 and Saccharopolyspora Species (cont.)

Characteristic	A83543.1	<u>S. erythraea</u>	<u>S. hirsuta</u>	<u>S. hirsuta</u> subsp. <u>taberi</u>	<u>S. hirsuta</u> subsp. <u>kobensis</u>
Acid produced from:					
melibiose	-	+	ND	-	ND
raffinose	-	+	+	+	+
rhamnose	-	+	+	+	-
salicin	-	+	-	-	ND
sucrose	-	+	+	+	+
xylose	-	+	+	-	-
lactose	-	-	+	-	ND
α -Me-D-glucoside	-	-	+	+	ND
sorbitol	-	-	+	-	-
inulin	-	ND	+	ND	+

* ND = not done

The comparisons made indicate that A83543.1 differs sufficiently from the previously described species of Saccharopolyspora to be a new species of Saccharopolyspora for which the name Saccharopolyspora spinosa has been selected. The name spinosa reflects the spiny spore ornamentation of this species.

The A83543.3, A83543.4 and A83543.5 strains are sufficiently similar macroscopically to the A83543.1 strain to be classified as strains of Saccharopolyspora spinosa. The four strains differ in the amount of 55 A83543 they produce. The A83543.3 strain produces approximately four-fold more A83543 than the A83543.1 strain; and the A83543.4 and A83543.5 strains produce approximately eight to nine-fold more than the A83543.1 strain produces.

As is the case with other organisms, the characteristics of the A83543-producing cultures of this invention, *Saccharopolyspora spinosa* NRRL 18395, NRRL 18537, NRRL 18538 and NRRL 18539, continue to be subject to variation. Thus, mutants of these strains may be obtained by physical and chemical methods known in the art. For example, other strains can be obtained by treatment with chemicals such as N-methyl-N'-nitro-N-nitrosoguanidine. Natural and induced mutants of the *Saccharopolyspora spinosa* NRRL 18395, NRRL 18537, NRRL 18538 and NRRL 18539 strains which retain the characteristic of producing a recoverable amount of A83543 are part of this invention.

The culture medium used to grow the *Saccharopolyspora spinosa* cultures can be any one of a number of media. For economy in production, optimal yield, and ease of product isolation, however, certain culture media are preferred. Thus, for example, preferred carbon sources in large-scale fermentation are glucose and maltose, although ribose, xylose, fructose, galactose, mannose, mannitol, soluble starch, potato dextrin, methyl oleate, oils such as soybean oil and the like can also be used.

Preferred nitrogen sources are cottonseed flour, peptonized milk and digested soybean meal, although fish meal, corn steep liquor, yeast extract, enzyme-hydrolyzed casein, beef extract, and the like can also be used.

Among the nutrient inorganic salts which can be incorporated in the culture media are the customary soluble salts capable of yielding zinc, sodium, magnesium, calcium, ammonium, chloride, carbonate, sulfate, nitrate and like ions.

Essential trace elements necessary for the growth and development of the organism should also be included in the culture medium. Such trace elements commonly occur as impurities in other constituents of the medium in amounts sufficient to meet the growth requirements of the organism.

Usually if foaming is a problem, small amounts (i.e., 0.2 ml/L) of an antifoam agent such as polypropylene glycol may be added to large-scale fermentation media. In the case of the A83543-producing cultures, however, conventional defoamers inhibit A83543 production. Foaming can be controlled by including soybean oil or pluronic L-101 (BASF) in the medium (1-3%). Additional oil may be added if foaming develops.

The percentage of a particular A83543 component may be varied by media changes. For example, adding valine or isobutyric or propionic acids increases the percentage of A83543D produced.

For production of substantial quantities of A83543, submerged aerobic fermentation in stirred bioreactors is preferred. Small quantities of A83543 may be obtained by shake-flask culture. Because of the time lag in production commonly associated with inoculation of large bioreactors with the spore form of the organism, it is preferable to use a vegetative inoculum. The vegetative inoculum is prepared by inoculating a small volume of culture medium with the spore form or mycelial fragments of the organism to obtain a fresh, actively growing culture of the organism. The vegetative inoculum is then transferred to a larger bioreactor. The vegetative inoculum medium can be the same as that used for larger fermentations, but other media are also suitable.

A83543 is produced by the A83543-producing organisms when grown at temperatures between about 24° and about 33°C. Optimum temperatures for A83543 production appear to be about 28-30°C.

As is customary in submerged aerobic culture processes, sterile air is blown into the vessel from the bottom while the medium is stirred with conventional turbine impellers. In general, the aeration rate and agitation rate should be sufficient to maintain the level of dissolved oxygen at or above 35%, and preferably at or above 50%, of air saturation with an internal vessel pressure of 0.34 atmospheres.

Production of the A83543 components can be followed during the fermentation by testing extracts of the broth. HPLC, using a system as described in Example 1, is a useful assay for this purpose.

Following their production under submerged aerobic fermentation conditions, the A83543 components can be recovered from the fermentation medium by methods used in the art. The A83543 produced during fermentation of the A83543-producing organism occurs in both the mycelia and the broth. A83543 appears to be lipophilic. Thus, if a substantial amount of oil is used in the fermentation, whole broth extraction is more efficient. If only small amounts of oil are used, the major portion of the A83543 is in the mycelia. In that case, more efficient recovery of A83543 is accomplished by initially filtering the medium to separate the broth from the mycelial mass (the biomass).

A83543 can be recovered from the biomass by a variety of techniques. A preferred technique involves washing the separated biomass with water to remove remaining broth, mixing the biomass with a polar solvent in which A83543 is soluble, e.g. methanol or acetone, separating and concentrating the solvent, extracting the concentrate with a non-polar solvent and/or adsorbing it onto a reverse-phase silica gel adsorbent such as RP-C8 or RP-C18 or a high porous polymer like HP-20 and the like.

The active material is eluted from the adsorbent with a suitable solvent such as, for example, acetonitrile:methanol mixtures containing small amounts of THF.

A83543 can be separated into individual components A83543A, A83543B, A83543C, A83543D, A83543E, A83543F, A83543G, A83543H, and A83543J and A83543A pseudoaglycone by similar procedures. A preferred separation procedure involves reverse-phase silica-gel (C₁₈ or C₈) chromatography.

Alternatively, the culture solids, including medium constituents and mycelium, can be used without extraction or separation, but preferably after removal of water, as a source of A83543. For example, after production of A83543, the whole fermentation broth can be dried by lyophilization, by drum-drying, or by azeotropic distillation and drying. The dried broth can then be used directly, e.g. by mixing it directly into feed premix

10 Insecticide and Miticide Activity

The compounds of this invention are useful for the control of insects and mites. The present invention is also directed, therefore, to methods for inhibiting an insect or mite which comprises applying to the locus of the insect or mite an insect- or mite-inhibiting amount of an A83543 compound.

15 The A83543 compounds show activity against a number of insects and mites. More specifically, the compounds show activity against Southern armyworm, which is a member of the insect order Lepidoptera. Other typical members of this order are codling moth, cutworms, clothes moths, Indianmeal moth, leaf rollers, corn earworm, cotton bollworm, European corn borer, imported cabbageworm, cabbage looper, pink bollworm, bagworms, Eastern tent caterpillar, sod webworm and fall armyworm

20 The compounds also show activity against cotton aphid, which is a member of the insect order Homoptera. Other members of the Homoptera include leafhoppers, planthoppers, pear psylla, apple sucker, scale insects, whiteflies and spittle bugs, as well as a number of other host-specific aphid species

In addition, the A83543 compounds show activity against stable flies, blow flies and mosquitoes, which are members of the insect order Diptera. Another typical member of this order is the common house fly.

25 The A83543 compounds are useful for reducing populations of insects and mites, and are used in a method of inhibiting an insect or mite population which comprises applying to a locus of the insect or mite an effective insect- or mite-inactivating amount of an A83543 compound.

The "locus" of insects or mites refers to the environment in which the insects or mites live or where their eggs are present, including the air surrounding them, the food they eat, or objects which they contact.

30 For example, plant-ingesting insects or mites can be controlled by applying the active compound to plant parts which the insects or mites eat or inhabit, particularly the foliage.

It is contemplated that the compounds might also be useful to protect textiles, paper, stored grain, or seeds by applying an active compound to such substances.

35 The term "inhibiting an insect or mite" refers to a decrease in the number of living insects or mites or to a decrease in the number of viable insect or mite eggs. The extent of reduction accomplished by a compound depends, of course, upon the application rate of the compound, the particular compound used, and the target insect or mite species. At least an insect-inactivating or mite-inactivating amount should be used.

40 The terms "insect-inactivating amount" and "mite-inactivating amount" are used to describe the amount which is sufficient to cause a measurable reduction in the treated insect or mite population. Generally an amount in the range from about 1 to about 1000 ppm (or 0.01 to 1 kg/ha) of active compound is used.

In one preferred embodiment, the present invention is directed to a method for inhibiting a susceptible insect of the order Lepidoptera which comprises applying to a plant an effective insect-inactivating amount of an A83543 compound in accordance with the present invention.

45 Another preferred embodiment of the invention is directed toward a method for inhibiting biting flies of the order Diptera in animals which comprises administering an effective pest-inhibiting amount of an A83543 compound orally or parenterally to the animal.

MITE/INSECT SCREEN

50

The A83543 compounds were tested for miticidal and insecticidal activity in the following mite/insect screen.

Each test compound was formulated by dissolving the compound in an acetone/alcohol (1:1) mixture containing 23 g of "Toximul R" (sulfonate/nonionic emulsifier blend) and 13 g of "Toximul S" (sulfonate/nonionic emulsifier blend) per liter. These mixtures were then diluted with water to give the indicated concentrations.

Two-spotted spider mites (*Tetranychus urticae* Koch) and cotton or melon aphids (*Aphis gossypii* Glover) were introduced on squash cotyledons and allowed to establish on both leaf surfaces. Other plants

in the same treatment pot were left uninfested. The leaves were then sprayed with 5 mL of test solution using a DeVilbiss atomizing sprayer at 10 psi. Both surfaces of the leaves were covered until runoff and then allowed to dry for one hour. Two uninfested leaves were then excised and placed into a Petri dish containing Southern armyworm (*Spodoptera eridania* Cramer).

5 Additional insects were evaluated using similar formulations and evaluation procedures, with the exceptions noted.

After standard exposure periods, percent mortality was evaluated. Results are reported in the tables infra. The following abbreviations are used:

Term	Insect/Mite	Scientific Name
BW	boll weevil	<i>Anthonomus grandis</i>
CA	cotton aphid	<i>Aphis gossypii</i>
CBW	cotton bollworm	<i>Heliothis zea</i>
15 CLH	corn leafhopper	<i>Dalbulus maidis</i>
CRW	Southern corn rootworm	<i>Diabrotica undecimpunctata howardi</i>
SAW	Southern armyworm	<i>Spodoptera eridania</i>
SM	twospotted spider mite	<i>Tetranychus urticae</i>

20 **Table X: Activity of A83543A Against**
Neonate CBW Larvae

<u>Treatment</u>	<u>Days^a</u>	<u>Rate (ppm)</u>	<u>% Inhibition^b</u>
Topical ^c	1	1.00	20.00
		5.00	100.00
		10.00	100.00
		50.00	100.00
		100.00	100.00
Diet ^d	4	1.00	30.00
		5.00	100.00
		10.00	100.00
		50.00	100.00
		100.00	100.00
Ova ^e	6	10.00	0.00
		50.00	0.00
		100.00	30.00

Table X (continued): Activity of A83543A Against
Neonate CBW Larvae

5

	<u>Treatment</u>	<u>Days^a</u>	<u>Rate (ppm)</u>	<u>% Inhibition^b</u>
10	Topical ^c	1	0.50	10.00
			1.00	40.00
			5.00	80.00
15			10.00	100.00
			50.00	100.00
			100.00	70.00
20	Diet	3	0.50	15.00
			1.00	45.00
			5.00	100.00
25			10.00	100.00
			50.00	100.00

30

^a Number of days between treatment and observation

^b Mean of two replicates tested

^c Treated with 1 mL of formulated A83543A

35

^d Diet is surface treated with A83543A,
 allowed to dry and infested

^e Eggs topically treated with A83543A and held until
 control eggs completely hatch

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Table XI

Percent Control of Neonate CBW Larvae by A83543 Components ¹				
Component ²	PPM			
	0.5	1	5	10
A	55 (d)	100 (a)	100 (a)	100 (a)
B	35 (e)	80 (c)	100 (a)	100 (a)
C	5 (g)	85 (bc)	93 (bc)	100 (a)
D	58 (d)	90 (ac)	100 (a)	100 (a)
E	28 (ef)	58 (d)	100 (a)	100 (a)
F	0 (g)	0 (g)	0 (g)	95 (ab)
G	0 (g)	0 (g)	20 (f)	80 (c)

1) Treatments with the same letter in parentheses are similar at the 0.05 level.

2) Topical pipet method with 20 larve/replicate and 4 replicates; one day between treatment and observation.

Table XII: Activity of A83543A
Against SAW Larvae

5

	<u>Stage</u>	<u>Treatment</u>	<u>Days^a</u>	<u>Rate (ppm)</u>	<u>% Inhibition^b</u>
10	Neonate	Foliar/ Bushbean ^c	3	1.00	10.00
				5.00	40.00
				10.00	100.00
				50.00	100.00
				100.00	100.00
15	Neonate	Topical ^d	3	1.00	0.00
				5.00	60.00
				10.00	100.00
				50.00	100.00
				100.00	100.00
20	Neonate	Foliar/ Bushbean ^e	4	0.50	0.00
				1.00	66.67
				5.00	100.00
				10.00	100.00
25				50.00	100.00
	Second Instar	Topical ^d	1	1.00	0.00
				5.00	0.00
				10.00	0.00
30				50.00	80.00
				100.00	100.00
	Second Instar	Foliar/ Bushbean ^d	4	1.00	0.00
35				5.00	0.00
				10.00	80.00
				50.00	80.00
				100.00	100.00
40	Third Instar	Topical ^e	2	1.00	0.00
				5.00	0.00
				10.00	13.33
				50.00	73.33
45	Third Instar	Foliar/ Bushbean ^e	4	0.50	0.00
				1.00	0.00
				5.00	40.00
				10.00	100.00
				50.00	100.00

50

55

Table XII (continued)

<u>Stage</u>	<u>Treatment</u>	<u>Days</u> ^a	<u>Rate (ppm)</u>	<u>% Inhibition</u> ^b
Fifth	Topical ^e	2	10.00	0.00
Instar			50.00	0.00
Fifth	Foliar/	4	10.00	0.00
Instar	Bushbean ^e		50.00	0.00

^a Days between treatment and observation

^b Mean of replicates tested

^c One replicate

^d Two replicates

^e Three replicates

^f Treated topically with 1 mL of formulated A83543A

Table XIII

Activity of A83543A Against Adult BW		
Treatment	Rate (ppm)	% Inhibition ^a
Topical ^b	1.00	0.00
	5.00	20.00
	10.00	20.00
	50.00	100.00
	100.0	100.00

^a One replicate; observed 3 days after treatment

^b Formulated A83543A (1 mL) poured over adult insects in a Petri plate

Table XIV: Activity of A83543A in Greenhouse
Tests Against Various Crop Pests

5	<u>Crop</u>	<u>Pest</u>	<u>Rate (ppm)</u>	<u>Inhibition^a</u>
	Corn	CRW	30	100
			15	50
10			7.5	10
			3.75	0
	Squash	SAW	250	100
			125	100
15			62.5	100
			31.25	55
			15.63	40
			7.8	20
			3.9	20
20			1.9	0
	Squash	SM	250	70
			125	40
			62.5	20
25			31.25	0
	Squash	CA	100	0
			50	0
30	Corn	CLH	200	90
			100	30
			50	0
35	Bushbean	SAW	100	100
			50	80
			25	40
			12.5	0
40	Bushbean	SM	100	100
			50	80
			25	30
			12.5	10
45			6.25	0

Table XIV (continued): Activity of A83543A in Greenhouse
Tests Against Various Crop Pests

50	<u>Crop</u>	<u>Pest</u>	<u>Rate (ppm)</u>	<u>Inhibition^a</u>
	Squash	CA	100	80
			50	40
			25	0
55	Corn	CRW	6	30
			3	0

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Table XV compares the effectiveness and persistence of A83543A treatment with that of methomyl in outdoor pot tests against Southern armyworm.

Table XV

Efficacy of A83543 Against <i>Spodoptera eridania</i> in Outdoor Pot Tests					
Treatment	Rate (ppm)	Days After Treatment ^a			
		1	5	7	14
A83543A	63	100	88	90	5
A83543A	125	100	88	95	90
A83543A	250	100	95	100	100
A83543A	500	100	100	100	100
methomyl	63	100	20	15	0
methomyl	125	100	35	40	20
methomyl	250	100	85	45	40
methomyl	500	100	90	85	60
None	0	0	0	0	0
None	0	0	0	0	0

^a Results given as % mortality

Table XVI summarizes the LC 50's, the lethal concentrations at which test compound inhibits 50% of test insect or mite, exhibited by A83543A as compared with that exhibited by the known insecticide methomyl.

Table XVI

LC 50'S for A83543 ^a		
Target	LC 50 (ppm)	
	A83543	methomyl
CRW	15	6
Grain Aphid	>250	5.4
SM	150	71.4
SAW/Foliar	1.3	11.4
CBW/Contact	0.6	14.5

^a Corn rootworm rate in soil by wt.; all others as spray concentration.

The A83543 components were active against yellow fever mosquito (*Aedes aegypti*) larvae in standard *in vitro* mosquito larvicide tests. Tables XVII and XVIII summarize the activity of the components in these tests.

Table XVII

5	In Vitro Activity of A83543B and A83543C Against First Instar Mosquito Larvae	
	A83543 Component	Minimal Inhibitory Concentration (mcg/mL) ^a
	A	0.016, 0.031 ^b
	B	0.016
	C	0.031

10 ^a Lowest concentration which showed 100% inhibition after 24 hr (on microtiter plates)
^b Tests on two lots

Table XVIII

20	Activity of A83543 Components vs. Fourth Instar Mosquito Larvae ^a	
	Component	Percent Control ^a
	A	60,70 ^b
	B	60
	C	60
	D	30
	E	80
25	F	0
	G	0

30 ^a At 24 hr when treated at 0.312 ppm
^b Results of two tests

Field Trials

35 A83543A was evaluated in field trials. In these trials, A83543A showed activity against imported cabbage worm and cabbage looper on sprouting broccoli and against a mixture of soybean loopers (75%) and fall armyworm (25%) on soybean.

Insecticidal Compositions

40 The compounds of this invention are applied in the form of compositions, which are also part of this invention. These compositions comprise an insect-or mite-inactivating amount of an A83543 compound and a phytologically acceptable inert carrier. The active component, i.e. the A83543 compound, may be present as 1) a single A83543 component, 2) a mixture of two or more components, 3) the separated A83543 mixture or 4) A83543 together with the dried portion of the fermentation medium in which it is produced, i.e. 45 the as crude, dried fermentation broth.

The compositions are either concentrated formulations which are dispersed in water for application, or are dust or granular formulations which are applied without further treatment.

50 The compositions are prepared according to procedures and formulae which are conventional in the agricultural chemical art, but which are novel and important because of the presence of one or more of the compounds of this invention.

The dispersions in which the compounds or crude dried material are applied are most often aqueous suspensions or emulsions prepared from concentrated formulations of the compounds or crude material. Such water-soluble, water-suspendable or emulsifiable formulations are either solids (usually known as wettable powders) or liquids (usually known as emulsifiable concentrates or aqueous suspensions).

55 Wettable powders, which may be compacted to form water dispersible granules, comprise an intimate mixture of the active compound, an inert carrier and surfactants. The concentration of the active compound is usually from about 1%, preferably 10%, to about 90% by weight. The inert carrier is usually chosen from among the attapulgite clays, the montmorillonite clays, the diatomaceous earths, or the purified silicates.

Effective surfactants, comprising from about 0.5% to about 10% of the wettable powder, are found among the sulfonated lignins, the condensed naphthalenesulfonates, the naphthalenesulfonates, the alkyl-benzenesulfonates, the alkyl sulfates, and non-ionic surfactants such as ethylene oxide adducts of alkyl phenols.

5 Emulsifiable concentrates of the compounds comprise a convenient concentration of a compound, such as from about 50 to about 500 grams per liter of liquid, equivalent to about 10% to about 50%, dissolved in an inert carrier which is either a water miscible solvent or a mixture of water-immiscible organic solvent and emulsifiers.

Useful organic solvents include aromatics, especially the xylenes, and the petroleum fractions, especially the high-boiling naphthalenic and olefinic portions of petroleum such as heavy aromatic naphtha. 10 Other organic solvents may also be used, such as the terpenic solvents including rosin derivatives, aliphatic ketones such as cyclohexanone, and complex alcohols such as 2-ethoxyethanol.

Suitable emulsifiers for emulsifiable concentrates are chosen from conventional nonionic surfactants, such as those mentioned above

15 Aqueous suspensions comprise suspensions of water-insoluble compounds of this invention, dispersed in an aqueous vehicle at a concentration in the range from about 5% to about 50% by weight. Suspensions are prepared by finely grinding the compound, and vigorously mixing it into a vehicle comprised of water and surfactants chosen from the same types discussed above. Inert ingredients, such as inorganic salts and synthetic or natural gums, may also be added to increase the density and viscosity of the aqueous vehicle. 20 It is often most effective to grind and mix the compound at the same time by preparing the aqueous mixture and homogenizing it in an implement such as a sand mill, ball mill, or piston-type homogenizer.

The compounds may also be applied as granular compositions, which are particularly useful for applications to the soil. Granular compositions usually contain from about 0.5% to about 10% by weight of the compound, dispersed in an inert carrier which consists entirely or in large part of clay or a similar 25 inexpensive substance.

Such compositions are usually prepared by dissolving the compound in a suitable solvent and applying it to a granular carrier which has been pre-formed to the appropriate particle size, in the range of from about 0.5 to 3 mm. Such compositions may also be formulated by making a dough or paste of the carrier and compound, and crushing and drying to obtain the desired granular particle size.

30 Dusts containing the compounds are prepared simply by intimately mixing the compound in powdered form with a suitable dusty agricultural carrier, such as kaolin clay, ground volcanic rock and the like. Dusts can suitably contain from about 1% to about 10% of the compound.

It is equally practical, when desirable for any reason, to apply the compound in the form of a solution in an appropriate organic solvent, usually a bland petroleum oil, such as the spray oils, which are widely used 35 in agricultural chemistry.

Insecticides and miticides are generally applied in the form of a dispersion of the active ingredient in a liquid carrier. It is conventional to refer to application rates in terms of the concentration of active ingredient in the carrier. The most widely used carrier is water.

40 The compounds of the invention can also be applied in the form of an aerosol composition. In such compositions the active compound is dissolved or dispersed in an inert carrier, which is a pressure-generating propellant mixture. The aerosol composition is packaged in a container from which the mixture is dispensed through an atomizing valve. Propellant mixtures comprise either low-boiling halocarbons, which may be mixed with organic solvents, or aqueous suspensions pressurized with inert gases or gaseous hydrocarbons.

45 The actual amount of compound to be applied to loci of insects and mites is not critical and can readily be determined by those skilled in the art in view of the examples provided. In general, concentrations of from 10 ppm to 5000 ppm of compound are expected to provide good control. With many of the compounds, concentrations of from 100 to 1000 ppm will suffice. For field crops, such as soybeans and cotton, a suitable application rate for the compounds is about 0.01 to 1 kg/ha, typically applied in 5-50 gal/A 50 of spray formulation.

The locus to which a compound is applied can be any locus inhabited by an insect or mite, for example, vegetable crops, fruit and nut trees, grape vines, and ornamental plants.

Because of the unique ability of mite eggs to resist toxicant action, repeated applications may be desirable to control newly emerged larvae, as is true of other known acaricides.

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Ectoparasiticide Activity

The A83543 compounds are also active against members of the insect order Diptera. Tables XIX - XXI summarize in vitro studies with A83543A and A83543D.

Table XIX

In vitro Efficacy of A83543A Against Black Blow Fly Larvae	
Dose Level (ppm)	Activity ¹
100	100
50	100
25	100
10	100
5	100
2	100
1	95
0.5	60
0.25	25

¹Activity = % mortality

Table XX

In vitro Efficacy of A83543A Against Adult Stable Fly		
Dose Level (ppm)	24 Hour Activity ¹	48 Hour Activity ¹
100	100	100
50	100	100
25	100	100
10	100	90
5	80	100
2	70	90
1	10	60
0.5	0	10

¹Activity = % mortality

Table XXI: In vitro Efficacy of A83543A and A83543D^{1,2}

ppm	A83543A				A83543D			
	ASF		LBF		ASF		LBF	
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
10	80	100	100	100	50	100	100	100
5	50	100	100	100	20	90	90	100
2.5	30	100	100	100	20	100	90	100
1.25	20	100	100	100	10	90	80	90
0.625	0	70	60	90	0	60	50	75
0.312	0	50	25	50	0	0	25	25

¹Percent insect mortality following 24 to 48 hrs. in vitro exposure to chemically added serum samples

²ASF = adult stable fly

LBF = blow fly larvae

ppm = parts per million

In in vivo tests, the A83543 compounds showed systemic insecticidal activity in guinea pigs and sheep against larval blow fly and adult stable fly with no obvious signs of toxicity. Representative compounds A83543A and A83543D have been tested in laboratory and target animals to determine the scope of activity. The following tests are illustrative.

Guinea Pig Systemic Test

Adult guinea pigs are used in this test system. The compounds to be tested are dissolved in aqueous polyvinyl pyrrolidone or in polyethylene glycol 200, and an appropriate amount of the solution is administered either orally or by intraperitoneal injection. Various doses of the compound are used, as set forth in the tables infra.

Blood is drawn from the guinea pigs at 30 minutes except as noted, and the samples of blood are centrifuged. Dental wicks are saturated with the blood serum, and then exposed in Petri dishes to adult stable flies; blow fly larvae are exposed in test tubes. After 24 and 48 hours, the insects are examined, and the number of dead are counted. The results of the tests are recorded as the percent of the insects which are killed.

Sheep Systemic Test

Tests are carried out in sheep using the test method described supra in the Guinea Pig Test description. Test compound is administered by intraperitoneal or intravenous injection or intraruminally; blood samples are drawn at 24 hours in these tests.

The results of guinea pig and sheep systemic tests using A83543A and A83543D are summarized in Tables XXII - XXV.

A83543A did not show anthelmintic activity in sheep treated by a single intraperitoneal or intraruminal dose of 50 mg/kg of body weight, or in mice experimentally infected with the intestinal nematode Nematospiroides dubius, when administered orally by a single gavage at 500 mg/kg.

Table XXII: In Vivo Insecticidal Activity of A83543A^a

Guinea Pig Systemic				Sheep Systemic			
mg/kg	LBF	ASF	Tox	mg/kg	LBF	ASF	Tox
10 (IP)	0	0	N	10 (IP)	0	0	N
20 (IP)	0	0	N	10 (IR)	0	0	N
30 (IP)	90	50	N	30 (IP)	80	0	N
50 (IP)	100	100	N	30 (IR)	100	30	N
50 (OR)	100	60*	N	50 (IP)	20	30	N
				50 (IR)	100	70	N
				2 x 25 (IR)	100	90	N
				1.0 (IV)	25	10	N

*5-hr blood sample; no 30-min sample taken

^aActivity measured as % mortality

ASF = adult stable fly

LBF = blow fly larvae

Tox = toxicity to the host animal
(N = none)

IP = intraperitoneal

IR = intraruminal

IV = intravenous

OR = oral

Table XXIII: Systemic Insecticidal Activity of A83543A and A83543D
in Guinea Pigs^a

Dose (mg/kg)	Route	Time Bled	A83543A		A83543D	
			% Activity		% Activity	
			LBF	ASF	LBF	ASF
10	IP	30 min	0	0	0	0
		5 hr	0	0	0	0
		24 hr	0	0	0	10
20	IP	30 min	0	0		
		5 hr	0	0		
		24 hr	0	0		
30	IP	30 min	90	50	0	0
		5 hr	50	0	0	0
		24 hr	30	0	0	0
50	IP	30 min	100	100	75	0
		5 hr	95	40	0	0
		24 hr	25	30	25	0
50	Oral	5 hr	100	60		
		24 hr	0	0		

^aLBF = blow fly larvae

ASF = adult stable fly

IP = intraperitoneal

Table XXIV: Insecticidal Assay of A83543A in the Blood of Sheep Challenged with Haemonchus contortus - 24-hour in vitro Exposure of Insects to Serum Samples Collected from Sheep^a

Treatment ^b	Initial Weight (kg)	Percent Insecticide Mortality ^c									
		Day 0		35 min		5 hr		Day 1		Day 2	
		L	A	L	A	L	A	L	A	L	A
50x1 (IP)	38.6	0	0	0	0	10	0	10	0	20	10
50x1 (IR)	31.8	0	0	0	10	40	10	90	0	20	0
25x2 (IR)	34.1	0	0	0	20	25	10	100	10	90	0
1x1 (IV)	25.9	0	0	25	10	10	0	0	0	0	0
Vehicle Control	44.5	0	0	0	0	0	0	0	0	0	0
Nontreated	30.9	0	0	0	0	0	0	0	0	0	0

^aL = larval blow fly

A = adult stable fly

IP = intraperitoneal

IR = intraruminal

IV = intravenous

^bAmount (mg/kg) times number of doses administered

^cTimes post-administration at which blood is drawn

Table XXV: Insecticidal Assay of A83543A in the Blood of Sheep Challenged with Haemonchus contortus - 48-hour in vitro Exposure of Insects to Serum Samples Collected from Sheep^a

Treatment ^b	Initial Weight (kg)	Percent Insecticide Mortality ^c														
		Day 0			35 min			5 hr			Day 1			Day 2		
		L	A		L	A		L	A		L	A		L	A	
50x1 (IP)	38.6	0	20	10	0	10	10	10	0	20	30	20	10	20	20	25
50x1 (IR)	31.8	0	0	0	10	75	30	100	60	100	70	100	20	40	20	25
25x2 (IR)	34.1	0	10	0	30	50	10	100	90	100	90	100	70	90	30	75
1x1 (IV)	25.9	0	0	25	10	10	20	0	0	0	0	0	0	0	0	0
Vehicle Control	44.5	0	10	0	0	0	20	0	10	0	0	0	20	0	0	0
Nontreated	30.9	0	40	0	0	0	0	0	0	0	0	10	0	0	0	0

^a L = larval blow fly
^b Amount (mg/kg) times number of doses administered
^c Times post-administration at which blood is drawn

A = adult stable fly
IP = intraperitoneal
IR = intraruminal
IV = intravenous

In in vitro tests against the sheep intestinal nematode Haemonchus contortus, A83543A killed 30% of the worms at a concentration of 100 ppm.

Ectoparasitocidal Methods

This invention also relates, therefore, to a method of controlling a population of insect ectoparasites which consume blood of a host animal which comprises administering to the host animal an effective amount of an A83543 compound. Insect ectoparasites include insect and acarina parasites. Administration to the animal may be by the dermal, oral or parenteral routes.

Parasitic insects and acarina include species that are bloodsucking as well as flesh eating and are parasitic during all of their life cycle or only part of their life cycle, such as only the larval or only the adult stage. Representative species include the following:

10	horse fly	<u>Tabanus spp.</u>
	stable fly	<u>Stomoxys calcitrans</u>
	black fly	<u>Simulium spp.</u>
	horse sucking louse	<u>Haematopinus asini</u>
	mange mite	<u>Sarcoptes scabiei</u>
15	scab mite	<u>Psoroptes equi</u>
	horn fly	<u>Haematobia irritans</u>
	cattle biting louse	<u>Bovicola bovis</u>
	shortnosed cattle louse	<u>Haematopinus eurysternus</u>
	longnosed cattle louse	<u>Linognathus vituli</u>
20	tsetse fly	<u>Glossina spp.</u>
	cattle follicle mite	<u>Demodex bovis</u>
	cattle tick	<u>Boophilus microplus</u> and <u>B. decoloratus</u>
	Gulf Coast tick	<u>Amblyomma maculatum</u>
	Lone Star tick	<u>Amblyomma americanum</u>
25	ear tick	<u>Otobius megnini</u>
	Rocky Mountain wood tick	<u>Dermacentor andersoni</u>
	screwworm fly	<u>Cochliomyia hominivorax</u>
	assassin bug	<u>Reduvius spp.</u>
	mosquito	<u>Culiseta inornata</u>
30	brown ear tick	<u>Rhipicephalus appendiculatus</u>
	African red tick	<u>Rhipicephalus evertsi</u>
	bont tick	<u>Amblyomma sp.</u>
	bont legged tick	<u>Hyalomma sp.</u>
	hog louse	<u>Haematopinus suis</u>
35	chigoe	<u>Tunga penetrans</u>
	body louse	<u>Haematopinus ovillus</u>
	foot louse	<u>Linognathus pedalis</u>
	sheep ked	<u>Melophagus ovinus</u>
	sheep scab mite	<u>Psoroptes ovis</u>
40	greenbottle fly	<u>Phaenicia sericata</u>
	black blow fly	<u>Phormia regina</u>
	secondary screw-worm	<u>Cochliomyia macellaria</u>
	sheep blow fly	<u>Phaenicia cuprina</u>
	bed bug	<u>Cimex lectularius</u>
45	Southern chicken flea	<u>Echidnophaga gallinacea</u>
	fowl tick	<u>Argas persicus</u>
	chicken mite	<u>Dermanyssus gallinae</u>
	scalyleg mite	<u>Knemidokoptes mutans</u>
	depluming mite	<u>Knemidokoptes gallinae</u>
50	dog follicle mite	<u>Demodex canis</u>
	dog flea	<u>Ctenocephalis canis</u>
	American dog tick	<u>Dermacentor variabilis</u>
	brown dog tick	<u>Rhipicephalus sanguineus</u>

The method of the invention may be used to protect economic and companion animals from ectoparasites. For example, the compounds may beneficially be administered to horses, cattle, sheep, pigs, goats, dogs, cats and the like, as well as to exotic animals such as camels, llamas, deer and other species which are commonly referred to as wild animals. The compounds may also beneficially be administered to poultry and other birds, such as turkeys, chickens, ducks and the like. Preferably, the method is applied to

economic animals, and most preferably to cattle and sheep.

The rate, timing, and manner of effective application will vary widely with the identity of the parasite, the degree of parasitocidal attack, and other factors. Applications can be made periodically over the entire lifespan of the host, or for only a peak season of parasitic attack. In general, ectoparasite control is obtained with topical application of liquid formulations containing from about 0.00005 to 95.0% of compound, preferably up to 5%, and most preferably up to about 1% of compound. Effective parasite control is achieved at administration rates of from about 5 to about 100 mg/kg

The compounds are applied to host animals by conventional veterinary practices. Usually, the compounds are formulated into ectoparasitocidal compositions which comprise a compound and a physiologically-acceptable carrier. For example, liquid compositions may be simply sprayed on the animals for which ectoparasitocidal control is desired. The animals may also treat themselves by such devices as back rubbers, which may contain the toxicant compound in a cloth, for example, which the animal may walk against and contact. Dip tanks are also employed to administer the active agent to the host animal.

The present compounds display systemic ectoparasitocidal activity. The compounds have the ability to permeate the tissues of a host animal to which one of the compounds has been administered. Insect parasites which then consume blood or other living tissues of the host animal are thereby killed. The compounds are administered by dermal, oral or percutaneous routes.

This invention also relates to ectoparasitocidal compositions comprising a physiologically-acceptable inert carrier and an A83543 compound. These compositions may be prepared by methods known in the art, for example by dissolving the compound in one of many physiologically-acceptable adjuvants or diluents. Oral administration may be performed by mixing the compound in the animals' feed or drinking water, or by administering dosage forms such as tablets, capsules, boluses, or implants. Percutaneous administration is conveniently accomplished by subcutaneous, intraperitoneal and intravenous injection of an injectable formulation.

The compounds can be formulated for oral administration in the usual forms, such as drenches, tablets, or capsules. Such compositions, of course, require orally-acceptable inert carriers. The compounds can also be formulated as an injectable solution or suspension, for subcutaneous, dermal, intraruminal, intraperitoneal, intramuscular, or intravenous injection. In some applications, the compounds are conveniently formulated as one component of a standard animal feed. In this embodiment, it is usual to formulate the present compound first as a premix in which the compound is dispersed in a liquid or particulate solid carrier. The premix can contain from about 2 to 250 grams of compound per pound. The premix is in turn formulated into the ultimate feed by conventional mixing.

Since ectoparasitic attack generally takes place during a substantial portion of the host animal's lifespan, it is preferred to administer the compounds of the present invention in a form to provide sustained release over a period of time. Conventional procedures include the use of a matrix which physically inhibits dissolution, where the matrix is a waxy semisolid such as the vegetable waxes or a high molecular weight polyethylene glycol. A good way to administer the compounds is by means of a sustained-action bolus, such as those of Laby, U.S. Patent 4,251,506, and Simpson, British Patent 2,059,767. For such a bolus, the compound would be encapsulated in a polymeric matrix such as that of Nevin, U.S. Patent 4,273,920. Sustained release of the compounds of the present invention can also be achieved by the use of an implant such as from a silicone-containing rubber.

In order to illustrate more fully the operation of this invention, the following examples are provided:

EXAMPLE 1

A83543 HPLC Assay Method

The following analytical HPLC method is useful for monitoring the fermentation for production of A83543:

Centrifuge a sample of the whole broth, decant and remove the supernatant. Add enough methanol to the biomass to return the sample to the original volume, mix, and allow the mixture to stand a minimum of fifteen minutes. Centrifuge and filter the supernatant through a 0.45 μ filter.

Alternatively, the whole broth can be extracted with acetonitrile (1:4 broth:solvent) or acetone.

HPLC System:

Column Support:	8-x 100-mm column, silica gel-4 μ spherical C ₁₈ (Nova C18, Waters)
Mobile Phase:	CH ₃ CN/MeOH/H ₂ O (45/45/10) containing 0.05% ammonium acetate

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Flow Rate: 4 mL/min
 Detection: UV at 250 nm
 Retention Times: A83543A - 3.6 - 3.7 min
 A83543D - 4.4 - 4.5 min

EXAMPLE 2

Preparation of A83543 with Culture A83543.1

A. Shake-flask Fermentation

The culture *Saccharopolyspora spinosa* NRRL 18395, either as a lyophilized pellet or as a suspension maintained in liquid nitrogen, is used to inoculate a vegetative medium having composition A or B (medium B is preferred for large scale production):

VEGETATIVE MEDIUM A	
Ingredient	Amount (%)
Trypticase soy broth*	3.0
Yeast extract	0.3
MgSO ₄ ·7H ₂ O	0.2
Glucose	0.5
Maltose	0.4
Deionized water	q.s 1 liter
No pH adjustment	

*Baltimore Biological Laboratories

VEGETATIVE MEDIUM B	
Ingredient	Amount (%)
Enzyme-hydrolyzed casein*	3.0
Yeast extract	0.3
MgSO ₄ ·7H ₂ O	0.2
Glucose	1.0
Deionized water	q s. 1 L
pH 6.2, adjust to 6.5 with NaOH	

*NZ Amine A, Sheffield Products, P.O. Box 638 Norwich, NY 13815

Slants or plates can be prepared by adding 2.5% agar to vegetative seed medium A or B. The inoculated slant is incubated at 30 °C. for from about 10 to 14 days. The mature slant culture is scraped with a sterile tool to loosen the spores and remove and macerate the mycelial mat. About one-fourth of the loosened spores and culture growth thus obtained is used to inoculate 50 mL of a first-stage vegetative seed medium. Alternatively, the first-stage medium may be inoculated from a liquid nitrogen ampoule.

When the culture is maintained in liquid nitrogen, ampoules are prepared using equal volumes of vegetative culture (48-72 hr incubation, 30 °C) and suspending medium. The suspending medium contains lactose (100 g), glycerol (200 mL) and deionized water (q.s. to 1 L).

A liquid nitrogen ampoule is used to inoculate 100 mL of vegetative medium in 500-mL Erlenmeyer flasks (or 50 mL medium in 250-mL flasks). The cultures are incubated at 30 °C for 48 hours on a shaker orbiting in a two-inch (5.08 cm) circle at 250 rpm.

The incubated culture (5% v/v inoculum) is used to inoculate 100 mL of a production medium having the following composition.

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PRODUCTION MEDIUM I	
Ingredient	Amount (%)
Glucose	4
Vegetable protein, partially hydrolyzed enzymatically*	1.5-3
Cottonseed flour**	1.0
CaCO ₃ (reagent or technical grade)	0.3
Soybean oil	1.0
Tap water	q.s. 1 liter
(Presterilization pH adjusted to 7.0 with NaOH)	

*Sheftone H, Sheffield Products

**Proflo, Traders Protein, P.O. Box 8407, Memphis, TN 38108

The inoculated production medium is incubated in 500-mL Erlenmeyer flasks at 28-30°C. for 6 to 8 days on a shaker orbiting in a two-inch circle at 250 rpm.

B. Stirred Bioreactor Fermentation

In order to provide a larger volume of inoculum, 10 mL of incubated first-stage medium, prepared as described in Section A, is used to inoculate 400 mL of a second-stage vegetative medium having the same composition as that of the first-stage vegetative medium. This second-stage medium is incubated in a 2-L wide-mouth Erlenmeyer flask for about 48 hours at 30°C. on a shaker orbiting in a two-inch circle at 250 rpm.

Incubated second-stage vegetative medium (2 L) thus prepared is used to inoculate 80 to 115 liters of sterile production medium, prepared as described in Section A. Additional soybean oil is added to control foaming, if needed.

The inoculated production medium is allowed to ferment in a 165-L stirred bioreactor for 5 to 8 days at a temperature of 28°C. The airflow and agitator speed in the stirred vessel are computer controlled to maintain a dissolved oxygen level at or above 50% of air saturation.

EXAMPLE 3

Isolation of A83543A, B, C and D

Fermentation broth (225 liters), prepared as described in Example 2, was filtered using a filter aid (1% Hyflo), and the separated biomass was washed with water (~ 50 L). The biomass was then agitated with methanol (~ 100 L) for about one hour and filtered.

The methanol filtrate was concentrated to a volume of about 1 liter. The concentrate was extracted three times with diethyl ether (1 L each). The combined ether extracts were concentrated to a volume of about 200 mL.

A portion of the concentrate (8 mL) was chromatographed on a silica gel column (RP-8 Lobar, size B, E.M. Science, a Division of E.M. Industries, Inc.).

This procedure was repeated for a total of 12 runs (cycles). The instrumental set-up and execution procedure to perform the preparative chromatography in an "Autoprep" mode is described as follows:

A complete "Autoprep" HPLC system is comprised of three Rainin Rabbit HPX pumps, one pressure module, one Gilson Model 20 1B HPLC fraction collector, one ISCO-V⁴ absorbance detector and one Apple Macintosh Plus computer. The complete system is arranged according to instructions given in the Dynamax HPLC Method Manager manual from Rainin Instrument Company, Inc. The "Autoprep" HPLC configuration takes advantage of system automation to permit preparative separations to be run repetitively under virtually identical conditions with virtually identical results. Collecting and pooling corresponding fractions from multiple runs provides chromatographic capacity without the need for a large column.

Two solvent mixtures (A) and (B) are used in the isocratic mode at a flow rate of 8.0 mL/min.

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Solvent Systems		
Solvent	Amount (mL)	
	A	B
CH ₃ OH	95	100
CH ₃ CN	95	100
H ₂ O	10	-

5

10

The isocratic mixture used contains 60% of solvent B.

The runtime for each cycle is 28.0 minutes. The eluates from the first 16 minutes of each run are discarded. The following eluates are collected in 6 time-fractions, 2 minutes (16 mL) each.

The automatically combined fractions from each of the 12 cycles resulted in 6 final fractions (chromatographic cuts).

15

The presence of the active A83543 compounds is determined by analyzing each final fraction for mosquito larvae activity and also by analytical HPLC.

The active fractions are then combined according to their activity and HPLC profiles and are further purified, using the same "Autoprep" HPLC and solvent system, but with a high resolution, 21.4-mm x 25-cm preparative column (Rainin Dynamax), prepacked with 8 μ C-18 reversed phase silica gel, to give A83543 components A, B, C and D. Factors A and D crystallize from CH₃OH/H₂O.

20

The FD-MS of A83543C is shown in figure 5; and the FAB-MS of A83543B is shown in figure 9 of the drawings.

EXAMPLE 4

25

Purification of A83543A and D

Fermentation broth (10 L) was prepared as described in Example 2 Sect. A, except that 1) 200 mL of production medium was used in 1-L flasks; 2) soybean oil was omitted from the production medium, and 3) incubation was at 30° for 4-6 days. The broth was filtered. The filtrate, containing 4 mcg of A83543A/mL and no detectable quantities of A83543B, C, or D/mL, was discarded.

30

The biomass was washed with water and extracted for one hour with methanol. The extract (7 L) contained 72 mcg of A83543A/mL and 7 mcg of A83543D/mL.

35

The methanol extract was concentrated to a volume of 5 L, and added to HP-20 resin (150 mL, Mitsubishi Chemical Industries, Ltd., Japan) in water (2 L). This mixture was stirred for one hour.

The HP-20 resin mixture was then placed in a glass column. The initial effluent and the eluate using methanol:water (1:1, 1 L) were not active. The second eluate using methanol:water (7:3, 1 L) contained trace quantities of A83543A. The following eluate using methanol (1 L) contained the A83543A and A83543D activity.

40

The methanol eluate was concentrated and combined with 2 similar fractions from other work-ups and concentrated to dryness. The residue was dissolved in 75 mL of methanol:THF (4:1) and precipitated by addition into 10 volumes of acetonitrile. The mixture was filtered, and the filtrate was concentrated to dryness.

45

The residue was dissolved in methanol (25 mL) and applied to a 5.5-x 90-cm column of LH-20 Sephadex (Pharmacia LKB Biotechnology Inc., U.S.A.), prepared in methanol, collecting and analyzing 125 25-mL fractions, using the HPLC procedure described in Example 1.

Fractions containing the desired compounds were combined and concentrated. The residue was dissolved in methanol (10 mL) and applied to a 41.1-mm x 25-cm preparative column prepacked with 8 μ C-18 reversed phase silica gel (Rainin Dynamax).

50

The column was conditioned in methanol: acetonitrile:water (37.5:37.5:25). After sample application, the column was developed using a 180-min linear gradient of the following solvents.

55

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Solvent System		
Solvent	Amount (mL)	
	A	B
CH ₃ OH	37.5	45
CH ₃ CN	37.5	45
H ₂ O	25	10

5

10

The gradient was run from 100% A to 100% B, collecting 25-mL fractions.

Fractions containing A83543A were pooled, concentrated to dryness, dissolved in *t*-BuOH (5 mL) and lyophilized to give 778 mg of pure A83543A.

15

The IR spectrum of A83543A in CHCl₃ is shown in figure 1. The FD-MS spectrum of A83543A is shown in figure 4, the FAB-MS spectrum of A83543A is shown in figure 8 [FAB dispersent-dithiothreitol: dithioerythritol (5:1); and the EI-MS spectrum of A83543A is shown in figure 10.

20

Fractions containing A83543D were combined with D-containing fractions from 6 similar separations and were concentrated and chromatographed as described *supra*, using the same column but different solvents. The column was conditioned in methanol:acetonitrile: water(40:40:20). The solvent systems used to develop the column in a 180-min linear gradient operation were:

Solvent Systems		
Solvent	Amount (mL)	
	A	B
CH ₃ OH	40	95
CH ₃ CN	40	95
H ₂ O	20	10

25

30

Fractions containing A83543D were combined and concentrated. The residue was dissolved in *t*-BuOH (5 mL) and lyophilized to give 212 mg of A83543D.

The IR spectrum of A83543D in CHCl₃ is shown in figure 2; and the FD-MS spectrum of A83543D is shown in figure 6.

35

EXAMPLE 5

Isolation of A83543 Components E,F,G,H and J and the Pseudoaglycone of A

40

Fermentation broth (8 L), prepared using procedures similar to those described in Example 2, was treated as described in Example 4. Fractions from the LH-20 Sephadex column containing the desired compounds were combined with corresponding fractions from similar fermentations. Since components E, F, G, H, J and the pseudoaglycone of A are produced in very small quantities, numerous fermentations are required to provide sufficient quantities for further purification.

45

A pool of minor factors, prepared in this manner and containing approximately 1.6 grams of solid material, was applied to an HPLC column (Rainin Dynamax) prepacked with 8 micron C-18 reversed phase silica gel (ODS), as described in Example 4. The column was conditioned in CH₃OH:CH₃CN:H₂O (75:75:50), and the gradient was run from 100% of solvent (A) to 50% (B): with the following solvent systems:

Solvent Systems		
Solvent	Amount (%)	
	A	B
CH ₃ OH	75	95
CH ₃ CN	75	95
H ₂ O	50	10

50

55

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collecting 25-mL fractions. The following fractions were pooled:

Pool	Fractions
1	31 - 44
2	45 - 63
3	64 - 69
4	70 - 80
5	81 - 130
6	131 - 160

A portion of pool 5 (100 mL) was concentrated to a residue, dissolved in methanol (1 mL) and applied to a 21.4-mm x 250-mm HPLC column (Rainin Dynamax), as described in Example 3. The column was conditioned using solvent system (A) of the following solvent systems:

Solvent Systems		
Solvent	Amount (%)	
	A	B
CH ₃ OH	30	95
CH ₃ CN	30	95
H ₂ O(1N NH ₄ OAC,pH5.0)	40	—
H ₂ O	—	10

and developed using a 120-minute linear gradient from 100% solvent (A) to 50% of solvent (B), collecting 15-mL fractions at 7.5 mL/min. Elution was continued at 50% (B) for an additional 60 minutes. The following fractions were pooled:

Pool	Fraction	Component
1	37	F
2	38 - 48	E
3	52 - 63	B,G
4	65 - 70	H,J

These pools were combined with pools from other chromatographic runs using similar starting materials. The combined pools were further purified using column chromatography, as described *supra*; desalted on HP-20 resins, using standard techniques; and concentrated and lyophilized to give the following components:

Component	Quantity (mg)	Mol. Wt*	IR**
E	249	717	Fig. 13
F	4	717	Fig. 14
G	104	731	Fig. 15
H,J	87	717	Fig. 16
Pseudo A	288	590	Fig. 3

* by mass spectrometry

** KBr disc

The FD-MS and EI-MS spectra of A83543A pseudoaglycone are shown in Figures 7 and 11, respectively.

EXAMPLE 6**Preparation of A83543 with Culture A83543.3****5 A. Shake-flask Fermentation**

Using procedures like those of Example 2, Section A, the culture *Saccharopolyspora spinosa* NRRL 18537 is cultivated in shake flasks, but using vegetative medium C as follows:

10

VEGETATIVE MEDIUM C	
Ingredient	Amount
Enzyme-hydrolyzed casein*	30.0 g
Yeast extract	3.0 g
MgSO ₄ ·7H ₂ O	0.2 g
Glucose	10.0 g
Glycerol	20 mL
Deionized water	q.s 1 L
pH 6.6, adjust to 7.0 with NaOH	

15

20

*NZ Amine A

25 B. Stirred Bioreactor Fermentation

Liquid nitrogen ampoules of the culture are prepared as described in Example 2, using the general procedures of Sect B. One ampoule is used to inoculate a first-stage vegetative culture (50 mL of medium C in 250-mL flasks), which is incubated for about 48-72 hours. Incubated first-stage culture is used to inoculate (10-mL inoculum) a second-stage culture (400 mL of medium C in 2-L flasks), which is incubated for about 48 hrs. The incubated second-stage culture (5 L) is used to inoculate a production medium (115 L) having the following composition:

35

PRODUCTION MEDIUM II	
Ingredient	Amount (g/L)
Glucose	80
Peptonized milk*	20
Cottonseed flour**	20
CaCO ₃	5
Methyl oleate	30 mL/L
Tap water	q.s. to 1 L

40

45

* Peptonized Milk Nutrient, Sheffield Products; additional continuous feed, beginning the fourth day, at a rate of 5 mg/mL/day, may be used.

** Proflo

(Presterilization pH adjusted to 7.0 with NaOH)

50

The inoculated production medium is allowed to ferment in a 165-L stirred bioreactor for about 8 to 10 days, or longer, at a temperature of 30 °C. Dissolved oxygen (DO) levels are regulated by computerized systems set to maintain the DO level above 50% of air saturation as described in Example 2, Section B.

55

EXAMPLE 7**Preparation of A83543 with Culture A83543.5****5 A. Shake-flask Fermentation**

Using procedures like those of Example 2, Section A, the culture *Saccharopolyspora spinosa* NRRL 18539 is cultivated in shake flasks, using vegetative medium B.

10 B. Stirred Bioreactor Fermentation

Liquid nitrogen ampoules of the culture are prepared as described in Example 2, using the general procedures of Sect. B. One ampoule is used to inoculate a first-stage vegetative culture (50 mL of medium B in 250-mL flasks), which is incubated for 48 to 72 hours. Incubated first-stage culture is used to inoculate
 15 (10-mL inoculum) a second-stage culture (400 mL of medium B in 2-L flasks), which is incubated for about 48 hr. The incubated second-stage culture (2 L) is used to inoculate a production medium (115 L) having one of the following compositions:

20

PRODUCTION MEDIUM III	
Ingredient	Amount (g/L)
Glucose	80
Vegetable protein, partially hydrolyzed enzymatically*	20
25 Cottonseed flour**	10
CaCO ₃	5
Methyl oleate	30 mL/L
Tap water	q.s. to 1 L

30

* Shefftone H

** Proflo

(Presterilization pH adjusted to 7.0 with NH₄OH)

35

PRODUCTION MEDIUM IV	
Ingredient	Amount (Percent)
Glucose	8
40 Cottonseed flour*	3
Peptonized Milk**	2
Corn steep liquor	1
CaCO ₃ (tech. grade)	0.5
Methyl oleate	3.0
45 Tap water	q.s. to 1 L

45

* Proflo

** Peptonized Milk Nutrient

(Presterilization pH adjusted to 7.0 with NaOH)

50

The inoculated production medium is allowed to ferment in a 165-L stirred bioreactor for about 8 to 10 days, or longer, at a temperature of 30 °C. DO levels are regulated as described in Example 6.

55

EXAMPLE 8**Preparation of A83543 with Culture A83543.4**

- 5 Using procedures like those of Examples 2 and 7, the culture Saccharopolyspora spinosa NRRL 18538 is cultivated but using vegetative medium B and production medium III.

EXAMPLE 9

- 10 Fermentation broth is prepared as described in Example 8. A83543 is separated from the broth as follows:

1. Add an equal volume of acetone to the broth and filter, using a ceramic filter or filter aid with a filter press.
2. Adjust the filtrate broth to pH 10.
- 15 3. Add ethyl acetate ($\frac{1}{4}$ to $\frac{1}{2}$ the volume of the broth).
4. Recover the ethyl acetate extract by decanting off the immiscible aqueous portion; and concentrate the ethyl acetate extract to $\frac{1}{2}$ volume by vacuum.
5. Extract the concentrated ethyl acetate solution with aqueous 0.1 M tartaric acid ($\frac{1}{2}$ volume); and separate the phases
- 20 6. Remove the soluble ethyl acetate from the aqueous phase by vacuum (about 5%) evaporation. Concentrate the aqueous solution, using a reverse osmosis operation.
7. Adjust the concentrated aqueous solution to pH 10-11 with sodium hydroxide.
8. Separate the precipitate by filtration; wash with water; and dry under vacuum to give A83543.

EXAMPLE 10**A83543A Pseudoaglycone**

- 30 A sample of A83543 containing mostly component A (ca 100 mg) was dissolved into methanol (50 mL), water (2 mL) and concentrated HCl (3 mL). This solution was concentrated to dryness at 50°C. The residue was treated twice with diethyl ether (200 mL each), and the insoluble material was discarded. The combined ether solutions containing the crude pseudoaglycone were concentrated to dryness. The residue was dissolved in methanol (20 mL) and purified, using the AUTOPREP-HPLC system described in Example 3, to give 20 mg of pure A83543A pseudoaglycone.

EXAMPLE 11

- A83543D pseudoaglycone is prepared from A83543D, using a procedure similar to that described in Example 10.

EXAMPLE 12

The following formulations are typical of the insecticidal compositions useful in this invention.

A. Aqueous Suspension	
A-83543A	12.5%
"Tergitol TMN-6" (nonionic surfactant)	1.0%
"Zeosyl 200" (silica)	1.0%
"AF-100" (silicon based antifoam agent)	0.2%
Xanthan solution (2%)	10.0%
"Makon 10" (10 moles ethyleneoxide nonylphenol surfactant)	9.0%
Tap water	66.3%

B. Emulsifiable Concentrate	
A83543D	12.4%
"Exxon 200" (naphthalene solvent)	83.6%
"Toximul H" (nonionic/anionic surfactant blend)	2.0%
"Toximul D" (nonionic/anionic surfactant blend)	2.0%

EXAMPLE 13

The following exemplary compositions illustrate the sort of formulations used to practice the method of the present invention.

A. Feed Premix	
A83543A	10%
Rice hulls	85
Light mineral oil	5

B. Feed Premix	
A83543E	25%
Alfalfa meal	60
Powdered clay	5
Molasses	10

C. Suspension	
A83543A	30%
Naphthalenesulfonate salt	5
Nonionic surfactant	5
Fumed silica	1
Water	59

D. Drip-On Solution	
A83543A	20%
Nonionic surfactant	0.8
Propylene glycol	15
Water	64.2

E. Drip-On Suspension	
A83543B	10
Nonionic surfactant	1
Light mineral oil	89

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F. Injectable Solution	
A83543A	15%
Propylene glycol	85

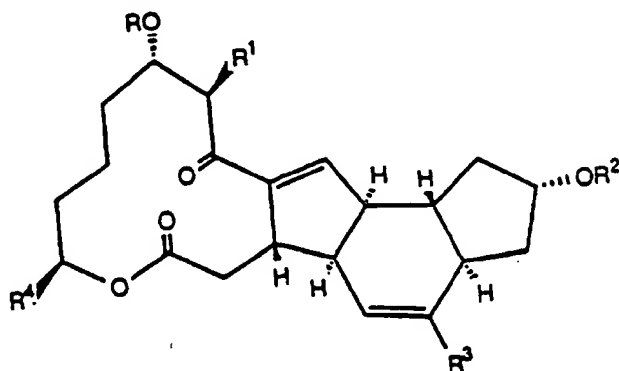
G. Injectable Suspension	
A83543C	25%
Propylene glycol	15
Water	60

H. Injectable Suspension	
A83543D	30%
Polyvinylpyrrolidone	2
Water	68

Claims

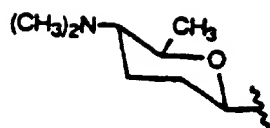
Claims for the following Contracting States : AT, BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. An A83543 compound of formula 1:

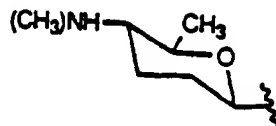


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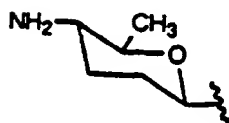
wherein R is H or a group selected from



(a)

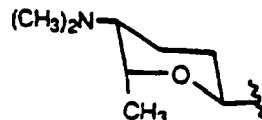


(b)

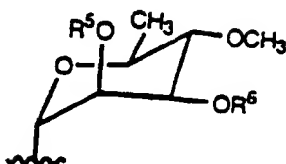


(c)

or



(d)

R² isR¹, R³, R⁵ and R⁶ are hydrogen or methyl;R⁴ is methyl or ethyl, or an acid addition salt thereof where R is other than hydrogen.

2. An A83543 compound of formula 1 as claimed in claim 1 wherein R, R¹, R³, R⁴, R⁵ and R⁶ are present in one of the following combinations:

R	R ¹	R ³	R ⁴	R ⁵	R ⁶
(a)	Me	H	Et	Me	Me
(b)	Me	H	Et	Me	Me
(c)	Me	H	Et	Me	Me
(a)	Me	Me	Et	Me	Me
(a)	Me	H	Me	Me	Me
(a)	H	H	Et	Me	Me
(d)	Me	H	Et	Me	Me
(a)	Me	H	Et	H	Me
(a)	Me	H	Et	Me	H
H	Me	H	Et	Me	Me
H	Me	Me	Et	Me	Me
H	Me	H	Me	Me	Me
H	H	H	Et	Me	Me
H	Me	H	Et	H	Me
H	Me	H	Et	Me	H

or an acid addition salt thereof when R is other than hydrogen.

3. Compound A83543A of Claim 2 wherein:

R is an (a) group; R¹, R⁵ and R⁶ = CH₃; R³ = H; and R⁴ = ethyl; or an acid addition salt thereof.

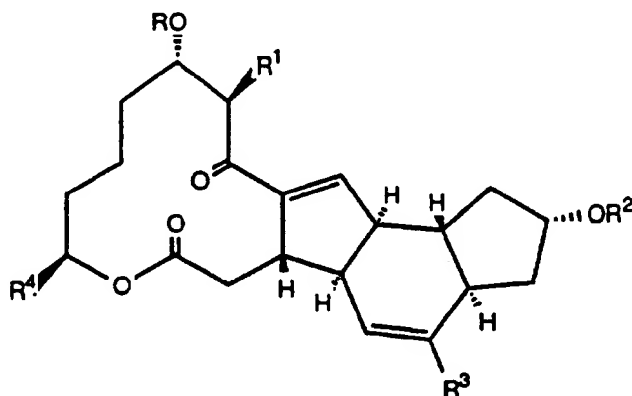
4. Compound A83543D of claim 2 wherein:

R is an (a) group; R¹, R³, R⁵ and R⁶ = CH₃; and R⁴ = ethyl; or an acid addition salt thereof.

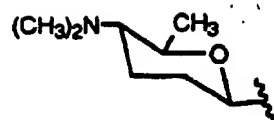
5. Fermentation product A83543, comprising A83543A and A83543D, which is produced by culturing a *Saccharopolyspora spinosa* strain selected from NRRL 18395, NRRL 18537, NRRL 18538 or NRRL 18539, or an A83543-producing mutant thereof, under submerged aerobic conditions in a suitable culture medium until a recoverable amount of A83543 is produced.
6. A process for producing an A83543 compound as claimed in any one of claims 1 to 4 which comprises cultivating a *Saccharopolyspora spinosa* strain selected from NRRL 18395, NRRL 18537, NRRL 18538 or NRRL 18539, or an A83543-producing mutant thereof, in a culture medium containing assimilable sources of carbon, nitrogen, and inorganic salts under submerged aerobic fermentation conditions until a recoverable amount of A83543 is produced.
7. A biologically purified culture of *Saccharopolyspora spinosa* selected from NRRL 18395, NRRL 18537, NRRL 18538, or NRRL 18539, or an A83543-producing mutant thereof.
8. An insecticide or miticide composition comprising an A83543 compound as claimed in any one of claims 1 to 4 and a phytologically-acceptable carrier
9. A method for inhibiting an insect or mite which comprises applying to the locus of the insect or mite an A83543 compound as claimed in any one of claims 1 to 4
10. An ectoparasiticial composition comprising a physiologically-acceptable inert carrier and an A83543 compound as claimed in any one of claims 1 to 4.

Claims for the following Contracting State: ES

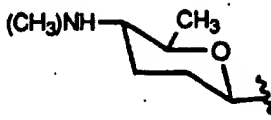
1. An insecticide or miticide composition comprising as an active ingredient from 1 to 90% by weight of an A83543 compound of formula 1:



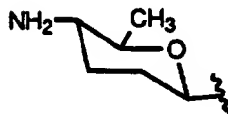
wherein R is H or a group selected from



(a)

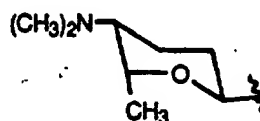


(b)

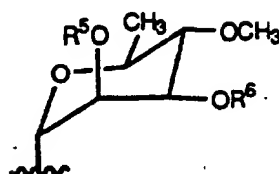


(c)

or



(d)

R² isR¹, R³, R⁵ and R⁶ are hydrogen or methyl;R⁴ is methyl or ethyl; or a phylogenically acceptable salt thereof where R is other than hydrogen; associated with one or more phylogenically acceptable carriers or diluents therefor.

2. An insecticide- or miticide formulation as claimed in claim 1 wherein the A83543 compound of formula is one in which R, R¹, R³, R⁴, R⁵ and R⁶ are present in one of the following combinations:

R	R ¹	R ³	R ⁴	R ⁵	R ⁶
(a)	Me	H	Et	Me	Me
(b)	Me	H	Et	Me	Me
(c)	Me	H	Et	Me	Me
(a)	Me	Me	Et	Me	Me
(a)	Me	H	Me	Me	Me
(a)	H	H	Et	Me	Me
(d)	Me	H	Et	Me	Me
(a)	Me	H	Et	H	Me
(a)	Me	H	Et	Me	H
H	Me	H	Et	Me	Me
H	Me	Me	Et	Me	Me
H	Me	H	Me	Me	Me
H	H	H	Et	Me	Me
H	Me	H	Et	H	Me
H	Me	H	Et	Me	H

or an acid addition salt thereof when R is other than hydrogen.

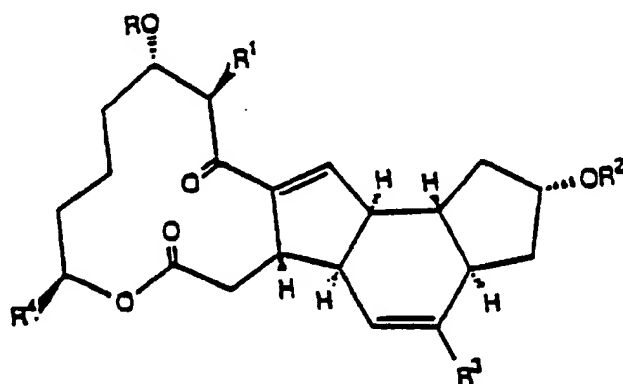
3. An insecticide- or miticide formulation as claimed in any one of claims 1 or 2 wherein the A83543 compound is one wherein: R is an (a) group; R¹, R⁵ and R⁶ = CH₃; R³ = H; and R⁴ = ethyl, or an acid addition salt thereof.

4. An insecticide- or miticide formulation as claimed in any one of claims 1 or 2 wherein the A83543 compound is one wherein: R is an (a) group; R¹, R³, R⁵ and R⁶ = CH₃; and R⁴ = ethyl; or an acid addition salt thereof.
5. A method for inhibiting an insect or mite which comprises applying to the locus of the insect or mite an effective amount of an A83543 compound, or a phytologically acceptable salt thereof, as defined in any one of claims 1 to 4.
6. An ectoparasiticide formulation comprising as an active ingredient from 0.00005 to 95% by weight of an A83543 compound of formula 1 as defined in claim 1 or a physiologically acceptable salt thereof associated with one or more physiologically-acceptable inert carriers or diluents therefor.
7. A process for producing an A83543 compound of formula 1 as defined in any one of claims 1 to 4 which comprises cultivating a *Saccharopolyspora spinosa* strain selected from NRRL 18395, NRRL 18537, NRRL 18538 or NRRL 18539, or an A83543-producing mutant thereof, in a culture medium containing assimilable sources of carbon, nitrogen, and inorganic salts under submerged aerobic fermentation conditions until a recoverable amount of A83543 is produced.
8. A biologically purified culture of *Saccharopolyspora spinosa* selected from NRRL 18395, NRRL 18537, NRRL 18538, or NRRL 18539, or an A83543-producing mutant thereof.

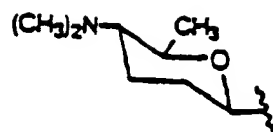
Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

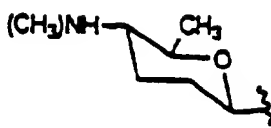
1. A83543-Verbindung der Formel (1):



worin R H oder eine Gruppe ist, die ausgewählt ist unter



(a)

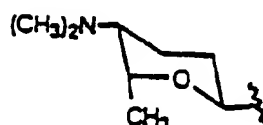


(b)

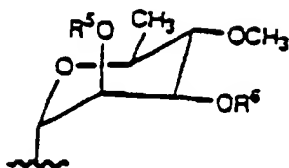


(c)

or



(d)

R²

ist

R¹, R³, R⁵ und R⁶ Wasserstoff oder Methyl ist,R⁴ Methyl oder Ethyl ist, oder ein Säureadditionssalz davon, wenn R kein Wasserstoff ist.

2. A83543-Verbindung der Formel (1) nach Anspruch 1, worin R, R¹, R³, R⁴, R⁵ und R⁶ in einer der folgenden Kombinationen vorhanden sind.

R	R ¹	R ³	R ⁴	R ⁵	R ⁶
(a)	Me	H	Et	Me	Me
(b)	Me	H	Et	Me	Me
(c)	Me	H	Et	Me	Me
(a)	Me	Me	Et	Me	Me
(a)	Me	H	Me	Me	Me
(a)	H	H	Et	Me	Me
(d)	Me	H	Et	Me	Me
(a)	Me	H	Et	H	Me
(a)	Me	H	Et	Me	H
H	Me	H	Et	Me	Me
H	Me	Me	Et	Me	Me
H	Me	H	Me	Me	Me
H	H	H	Et	Me	Me
H	Me	H	Et	H	Me
H	Me	H	Et	Me	H

oder ein Säureadditionssalz davon, wenn R nicht Wasserstoff ist.

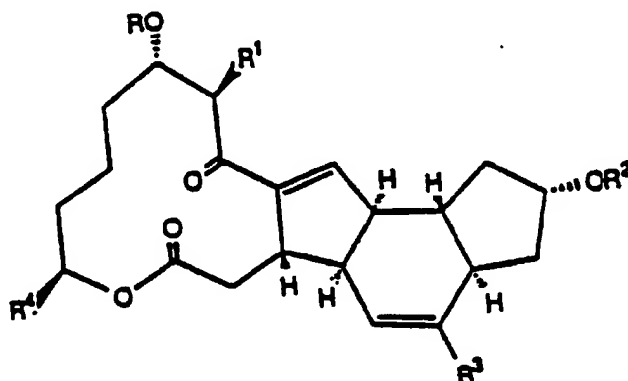
3. A83543A-Verbindung nach Anspruch 2, worin:

R eine (a)-Gruppe ist, R¹, R⁵ und R⁶ = CH₃, R³ = H und R⁴ = Ethyl oder ein Säureadditionssalz davon.

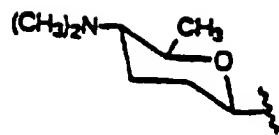
4. A83543D-Verbindung nach Anspruch 2, worin:
R eine (a)-Gruppe ist, R¹, R², R⁵ und R⁶ = CH₃ und R⁴ = Ethyl oder ein Säureadditionssalz davon.
5. Fermentationsprodukt A83543, welches A83543A und A83543D umfaßt, das durch Züchten eines Saccharopolyspora spinosa-Stamms hergestellt wird, der unter NRRL 18395, NRRL 18537, NRRL 18538 oder NRRL 18539 oder einem A83543-synthetisierenden Mutanten davon ausgewählt ist, unter aeroben Submers-Bedingungen in einem geeigneten Kulturmedium, bis eine gewinnbare Menge von A83543 synthetisiert wird.
6. Verfahren zur Synthese einer A83543-Verbindung nach einem der Ansprüche 1 bis 4, welches Züchten eines Saccharopolyspora spinosa Stamms umfaßt, der unter NRRL 18395, NRRL 18537, NRRL 18538 oder NRRL 18539 oder einem A83543-synthetisierenden Mutanten davon ausgewählt ist, in einem Kulturmedium, das assimilierbare Kohlenstoffquellen, Stickstoffquellen und anorganische Salze enthält, unter aeroben Submers-Fermentationsbedingungen, bis eine gewinnbare Menge an A83543 hergestellt wird.
7. Biologisch gereinigte Saccharopolyspora spinosa Kultur, welche unter NRRL 18395, NRRL 18537, NRRL 18538 oder NRRL 18539 oder einem A83543-synthetisierenden Mutanten davon ausgewählt ist
8. Insektizide oder mitizide Zusammensetzung, welche eine A83543-Verbindung nach einem der Ansprüche 1 bis 4 und einen phytologisch verträglichen Träger umfaßt
9. Verfahren zur Inhibierung eines Insekts oder einer Milbe, welches Auftragen einer A83543-Verbindung nach einem der Ansprüche 1 bis 4 auf den Ort des Insekts oder der Milbe umfaßt.
10. Ectoparasitizide Zusammensetzung, welche einen physiologisch verträglichen inerten Träger und eine A83543-Verbindung nach einem der Ansprüche 1 bis 4 umfaßt.

Patentansprüche für folgenden Vertragsstaat : ES

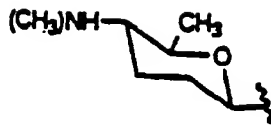
1. Insektizide oder mitizide Zusammensetzung, welche als einen Wirkstoff 1 bis 90 Gew.-% einer A83543-Verbindung der Formel (1) enthält:



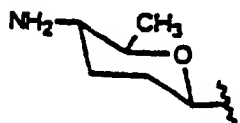
worin R H oder eine Gruppe ist, die ausgewählt ist unter



(a)

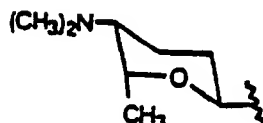


(b)

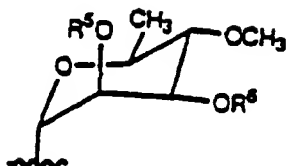


(c)

or



(d)

R²

ist

R¹, R³, R⁵ und R⁶ Wasserstoff oder Methyl ist,R⁴ Methyl oder Ethyl ist, oder ein phytologisch verträgliches Salz davon, wenn R kein Wasserstoff ist,

die mit einem oder mehreren phytologisch verträglichen Trägern oder Verdünnungsmitteln dafür assoziiert ist.

2. Insektizide oder mitizide Formulierung nach Anspruch 1, worin die A83543-Verbindung der Formel (1) eine Verbindung ist, bei der R, R¹, R³, R⁴, R⁵ und R⁶ in einer der folgenden Kombinationen vorhanden sind:

R	R ¹	R ³	R ⁴	R ⁵	R ⁶
(a)	Me	H	Et	Me	Me
(b)	Me	H	Et	Me	Me
(c)	Me	H	Et	Me	Me
(a)	Me	Me	Et	Me	Me
(a)	Me	H	Me	Me	Me
(a)	H	H	Et	Me	Me
(d)	Me	H	Et	Me	Me
(a)	Me	H	Et	H	Me
(a)	Me	H	Et	Me	H
H	Me	H	Et	Me	Me
H	Me	Me	Et	Me	Me
H	Me	H	Me	Me	Me
H	H	H	Et	Me	Me
H	Me	H	Et	H	Me
H	Me	H	Et	Me	H

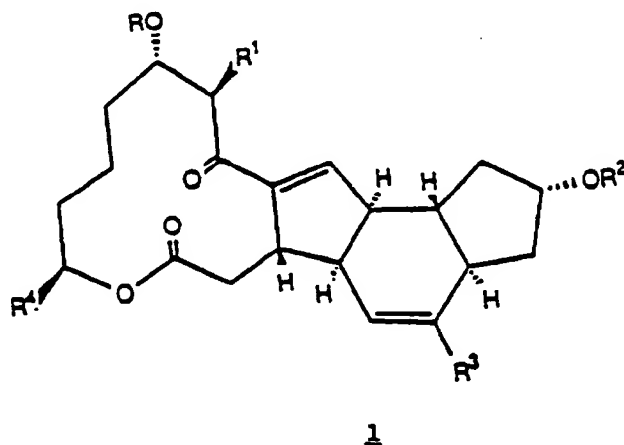
oder ein Säureadditionssalz davon, wenn R nicht Wasserstoff ist.

3. Insektizide oder mitizide Formulierung nach einem der Ansprüche 1 oder 2, worin die A83543-Verbindung eine Verbindung ist, worin: R eine (a)-Gruppe ist, R¹, R⁵ und R⁶ = CH₃, R³ = H und R⁴ = Ethyl oder ein Säureadditionssalz davon.
- 5 4. Insektizide oder mitizide Formulierung nach einem der Ansprüche 1 oder 2, worin die A83543-Verbindung eine Verbindung ist, worin: R eine (a)-Gruppe ist, R¹, R³, R⁵ und R⁶ = CH₃ und R⁴ = Ethyl oder ein Säureadditionssalz davon.
- 10 5. Verfahren zur Inhibierung eines Insekts oder einer Milbe, welches Auftragen einer wirksamen Menge einer A83543-Verbindung oder eines phytologisch verträglichen Salzes davon nach einem der Ansprüche 1 bis 4 auf den Ort des Insekts oder der Milbe umfaßt.
- 15 6. Ectoparasitizide Formulierung, welche als Wirkstoff von 0,00005 bis 95 Gew.-% einer A83543-Verbindung der Formel (1) nach Anspruch 1 oder eines physiologisch verträglichen Salzes davon assoziiert mit einem oder mehreren physiologisch verträglichen inerten Trägern oder Verdünnungsmitteln dafür enthält.
- 20 7. Verfahren zur Synthese einer A83543-Verbindung der Formel (1) nach einem der Ansprüche 1 bis 4, welches Züchten eines *Saccharopolyspora spinosa* Stamms umfaßt, der unter NRRL 18395, NRRL 18537, NRRL 18538 oder NRRL 18539 oder einem A83543-synthetisierenden Mutanten davon ausgewählt ist, in einem Kulturmedium, das assimilierbare Kohlenstoffquellen, Stickstoffquellen und anorganische Salze enthält, unter aeroben Submers-Fermentationsbedingungen, bis eine gewinnbare Menge an A83543 hergestellt ist.
- 25 8. Biologisch gereinigte *Saccharopolyspora spinosa* Kultur, welche unter NRRL 18395, NRRL 18537, NRRL 18538 oder NRRL 18539 oder einem A83543-synthetisierenden Mutanten davon ausgewählt ist.

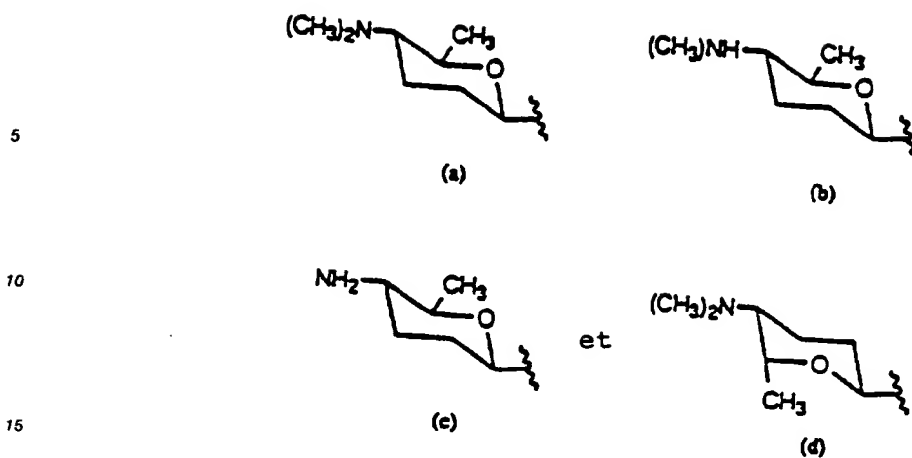
Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. Composé A83543 de formule 1 :



dans laquelle R représente un atome d'hydrogène ou un groupe choisi parmi :



20 R^2 représente un groupe de formule



30 R^1 , R^3 , R^5 et R^6 représentent chacun un atome d'hydrogène ou un groupe méthyle.
 R^4 représente un groupe méthyle ou éthyle,
ou un sel d'addition de ce composé avec un acide lorsque R n'est pas un atome d'hydrogène.

2. Composé A83543 de formule 1 selon la revendication 1, dans lequel R , R^1 , R^3 , R^4 , R^5 et R^6 répondent à l'une des combinaisons suivantes :

35

R	R^1	R^3	R^4	R^5	R^6
(a)	Me	H	Et	Me	Me
(b)	Me	H	Et	Me	Me
(c)	Me	H	Et	Me	Me
(a)	Me	Me	Et	Me	Me
(a)	Me	H	Me	Me	Me
(a)	H	H	Et	Me	Me
(d)	Me	H	Et	Me	Me
(a)	Me	H	Et	H	Me
(a)	Me	H	Et	Me	H
H	Me	H	Et	Me	Me
H	Me	Me	Et	Me	Me
H	Me	H	Me	Me	Me
H	H	H	Et	Me	Me
H	Me	H	Et	H	Me
H	Me	H	Et	Me	H

40

45

50

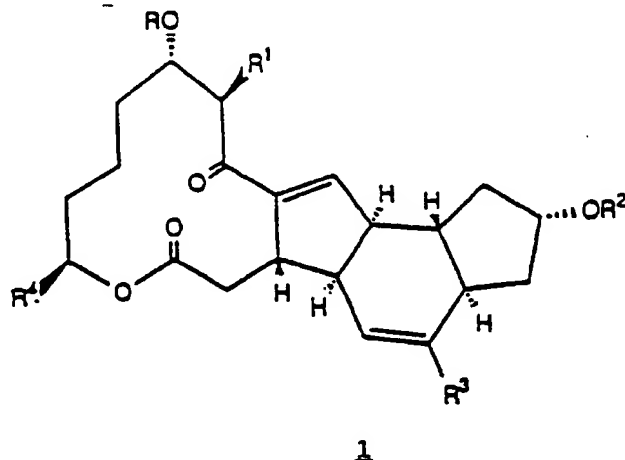
ou un sel d'addition de ce composé avec un acide lorsque R n'est pas un atome d'hydrogène.

3. Composé A83543A selon la revendication 2, dans lequel R représente un groupe (a), R^1 , R^5 et R^6 représentent chacun un groupe CH_3 , R^3 représente un atome d'hydrogène et R^4 représente un groupe éthyle, ou un sel d'addition de ce composé avec un acide.
- 55

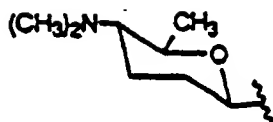
4. Composé A83543D selon la revendication 2, dans lequel R représente un groupe (a), R¹, R³, R⁵ et R⁶ représentent chacun un groupe CH₃, et R⁴ représente un groupe éthyle, ou un sel d'addition de ce composé avec un acide.
5. Produit de fermentation A83543, comprenant A83543A et A83543D, que l'on produit en cultivant une souche de *Saccharopolyspora spinosa*, choisie parmi NRRL 18395, NRRL 18537, NRRL 18538 et NRRL 18539, ou un mutant de cette souche, produisant A83543, dans des conditions de fermentation aérobie, en culture submergée, dans un milieu de culture approprié, jusqu'à ce qu'une quantité récupérable de A83543 soit produite.
6. Procédé de production d'un composé A83543 selon l'une quelconque des revendications 1 à 4, qui comprend la culture d'une souche de *Saccharopolyspora spinosa*, choisie parmi NRRL 18395, NRRL 18537, NRRL 18538 et NRRL 18539, ou d'un mutant de cette souche, produisant A83543, dans un milieu de culture contenant des sources assimilables de carbone et d'azote, et des sels minéraux, dans des conditions de fermentation aérobie, en culture submergée, jusqu'à ce qu'une quantité récupérable de A83543 soit produite.
7. Culture biologiquement purifiée de *Saccharopolyspora spinosa*, choisie parmi NRRL 18395, NRRL 18537, NRRL 18538 et NRRL 18539, ou d'un mutant de ces souches, produisant A83543.
8. Composition insecticide ou acaricide, comprenant un composé A83543 tel que revendiqué dans l'une quelconque des revendications 1 à 4, et un support acceptable du point de vue phytologique
9. Procédé pour inhiber un insecte ou un acarien, qui comprend l'application sur le lieu où se trouve l'insecte ou l'acarien, d'un composé A83543 tel que revendiqué dans l'une quelconque des revendications 1 à 4.
10. Composition ectoparasiticide, comprenant un support inerte, physiologiquement acceptable, et un composé A83543 tel que revendiqué dans l'une quelconque des revendications 1 à 4.

Revendications pour l'Etat contractant suivant : ES

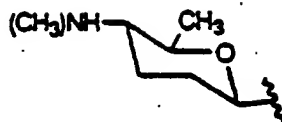
1. Composition insecticide ou acaricide, comprenant comme ingrédient actif de 1 à 90 % en poids d'un composé A83543 de formule 1 :



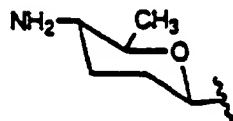
dans laquelle R représente un atome d'hydrogène ou un groupe choisi parmi :



(a)

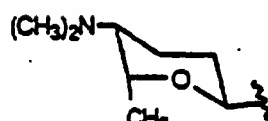


(b)



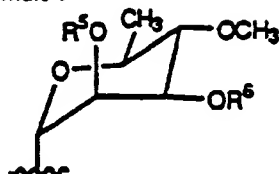
(c)

et



(d)

R² représente un groupe de formule :



R¹, R³, R⁵ et R⁶ représentent chacun un atome d'hydrogène ou un groupe méthyle,

R⁴ représente un groupe méthyle ou éthyle,

ou d'un sel de ce composé, acceptable du point de vue phytologique, lorsque R n'est pas un atome d'hydrogène, associé à un ou plusieurs supports ou diluants, acceptables du point de vue phytologique, pour ledit composé.

2. Composition insecticide ou acaricide selon la revendication 1, pour laquelle le composé A83543 de formule 1 est un composé dans lequel R, R¹, R³, R⁴, R⁵ et R⁶ répondent à l'une des combinaisons suivantes :

R	R ¹	R ³	R ⁴	R ⁵	R ⁶
(a)	Me	H	Et	Me	Me
(b)	Me	H	Et	Me	Me
(c)	Me	H	Et	Me	Me
(a)	Me	Me	Et	Me	Me
(a)	Me	H	Me	Me	Me
(a)	H	H	Et	Me	Me
(d)	Me	H	Et	Me	Me
(a)	Me	H	Et	H	Me
(a)	Me	H	Et	Me	H
H	Me	H	Et	Me	Me
H	Me	Me	Et	Me	Me
H	Me	H	Me	Me	Me
H	H	H	Et	Me	Me
H	Me	H	Et	H	Me
H	Me	H	Et	Me	H

ou un sel d'addition d'un tel composé avec un acide lorsque R n'est pas un atome d'hydrogène.

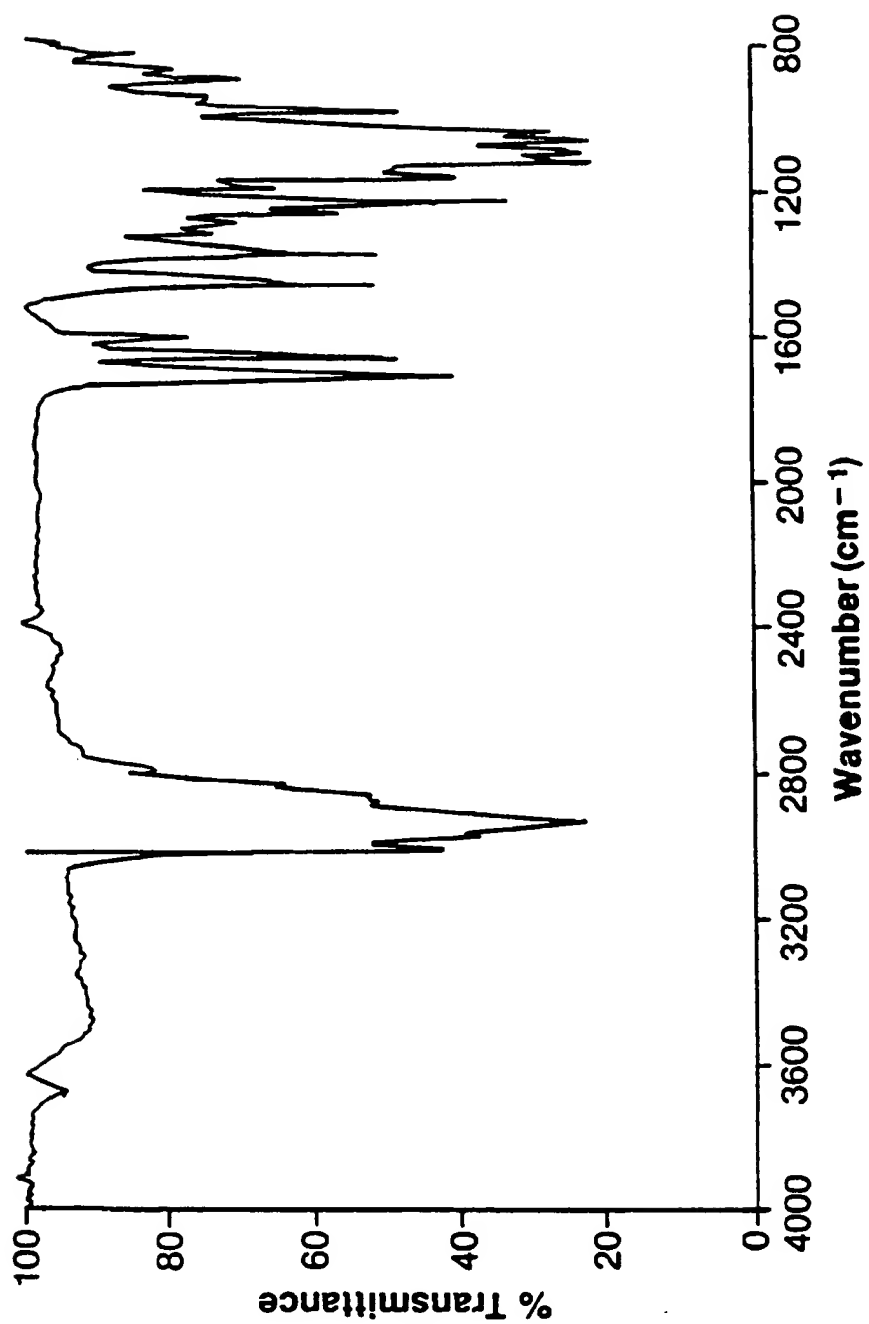
3. Composition insecticide ou acaricide selon la revendication 1 ou 2, pour laquelle le composé A83543 est un composé dans lequel R représente un groupe (a), R¹, R⁵ et R⁶ représentent chacun un groupe

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CH₃, R² représente un atome d'hydrogène et R⁴ représente un groupe éthyle, ou un sel d'addition d'un tel composé avec un acide.

4. Composition insecticide ou acaricide selon la revendication 1 ou 2, pour laquelle le composé A83543 est un composé dans lequel R représente un groupe (a), R¹, R³, R⁵ et R⁶ représentent chacun un groupe CH₃, et R⁴ représente un groupe éthyle, ou un sel d'addition d'un tel composé avec un acide.
5. Procédé pour inhiber un insecte ou un acarien, qui comprend l'application sur le lieu où se trouve l'insecte ou l'acarien, d'une quantité efficace d'un composé A83543 tel que défini dans l'une quelconque des revendications 1 à 4, ou d'un sel acceptable du point de vue phytologique d'un tel composé.
6. Composition ectoparasiticide comprenant, comme ingrédient actif, de 0,00005 % à 95 % en poids d'un composé A83543 de formule 1 tel que défini dans la revendication 1, ou d'un sel physiologiquement acceptable d'un tel composé, associé à un ou plusieurs supports ou diluants inertes, physiologiquement acceptables, pour l'edit composé ou l'edit sel.
7. Procédé de production d'un composé A83543 de formule 1 tel que défini dans l'une quelconque des revendications 1 à 4, qui comprend la culture d'une souche de Saccharopolyspora spinosa, choisie parmi NRRL 18395, NRRL 18537, NRRL 18538 et NRRL 18539, ou d'un mutant d'une telle souche, produisant A83543, dans un milieu de culture contenant des sources assimilables de carbone et d'azote, et des sels minéraux, dans les conditions de fermentation aérobie, en culture submergée, jusqu'à ce qu'une quantité récupérable de A83543 soit produite
8. Culture biologiquement purifiée de Saccharopolyspora spinosa, choisie parmi NRRL 18395, NRRL 18537, NRRL 18538 et NRRL 18539, ou d'un mutant de ces souches, produisant A83543.

FIG.1



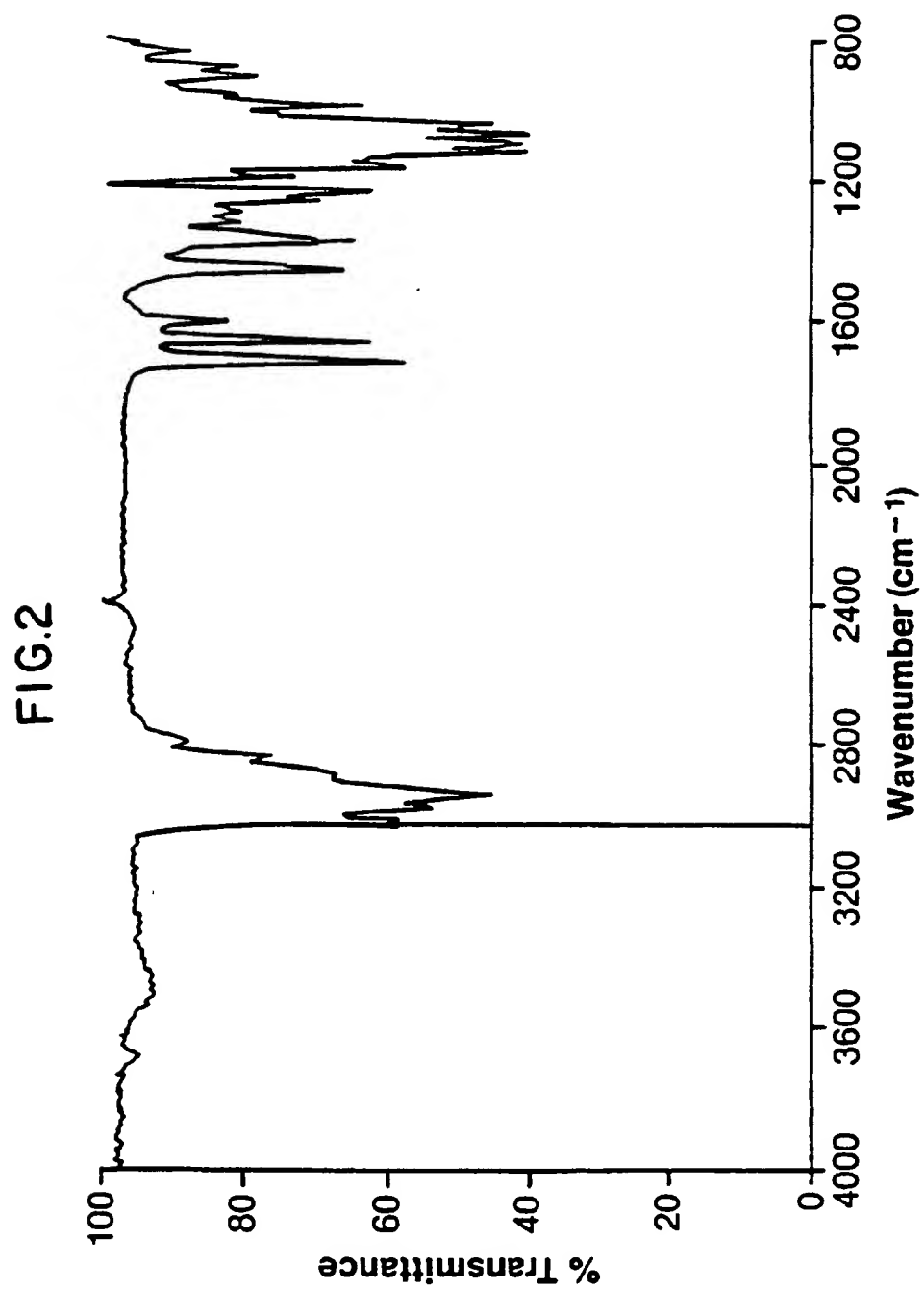


FIG.3

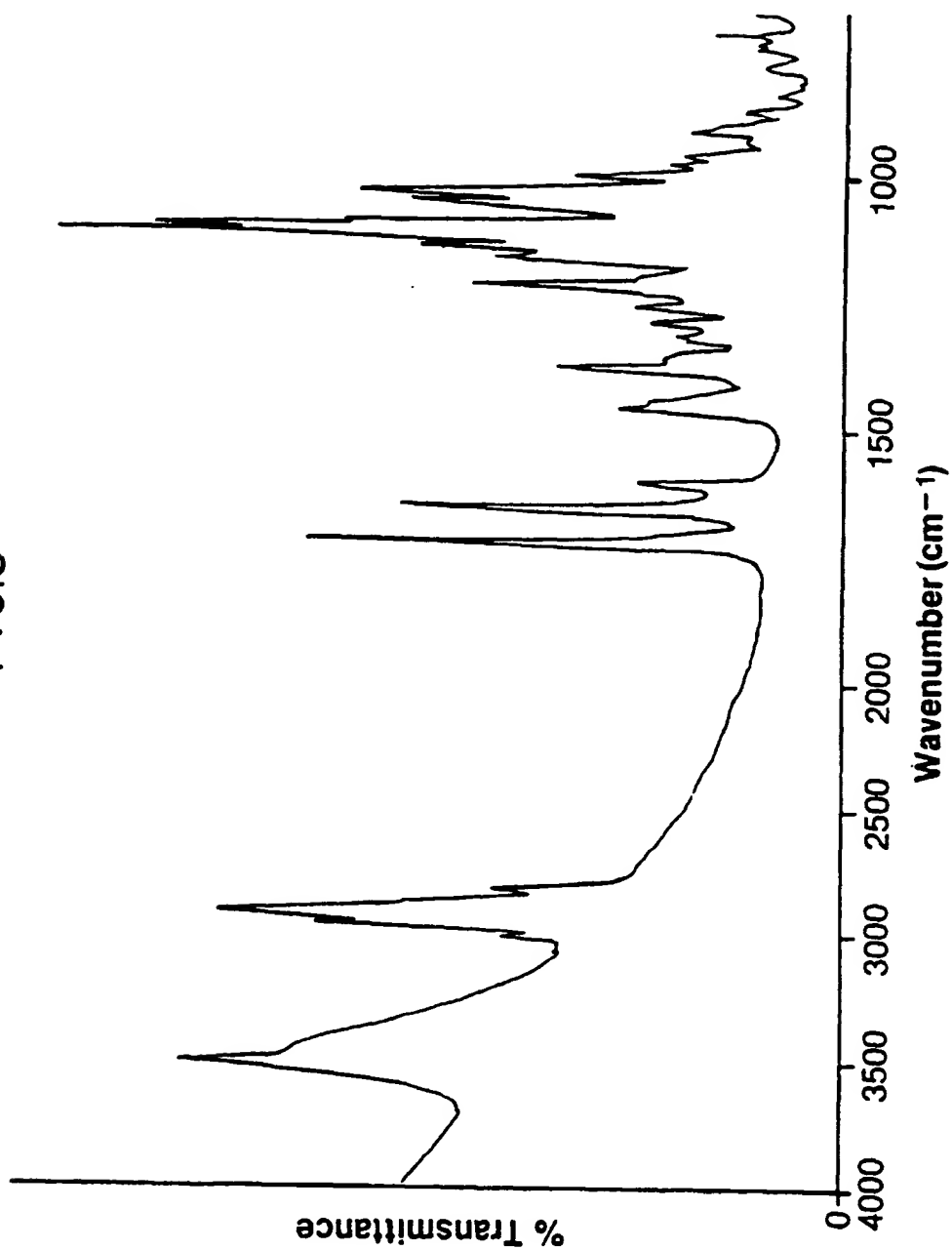


FIG. 4

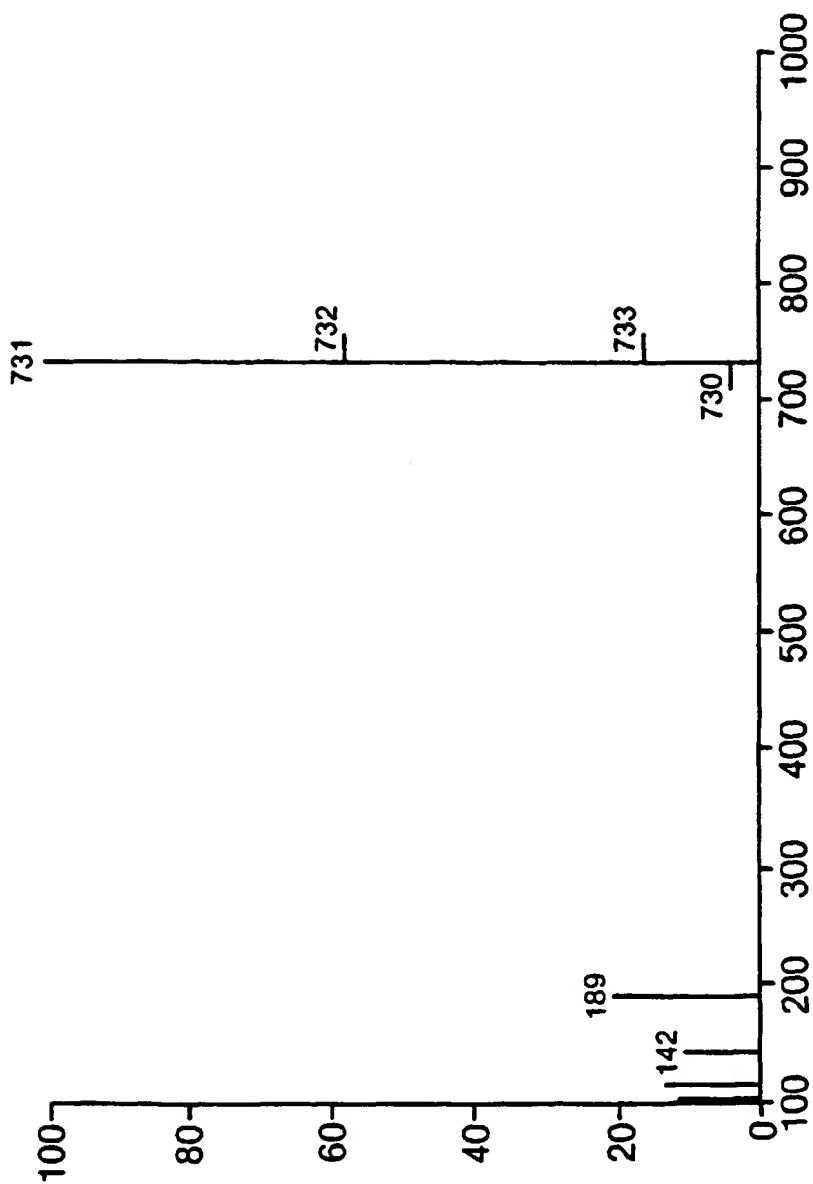


FIG.5

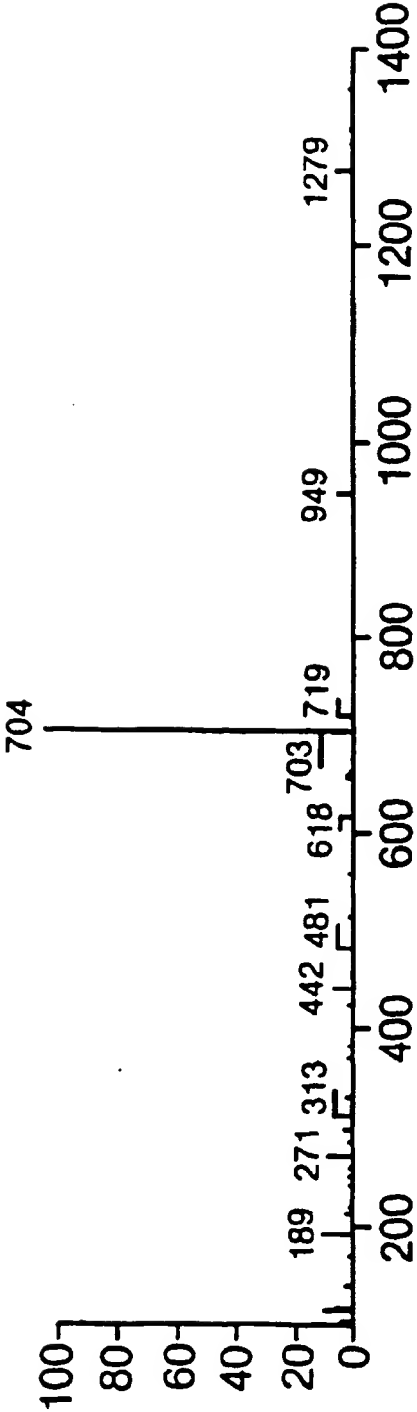


FIG. 6

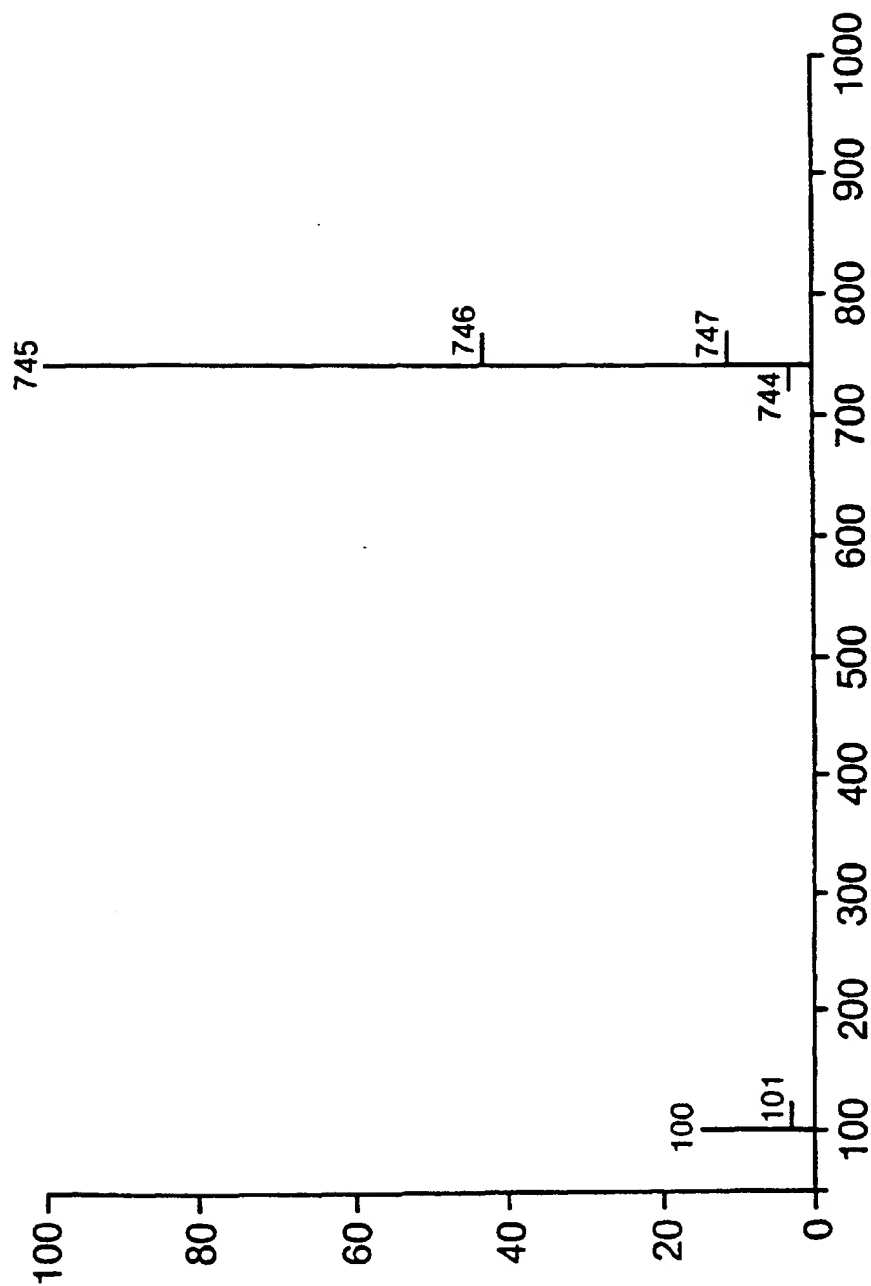


FIG. 7

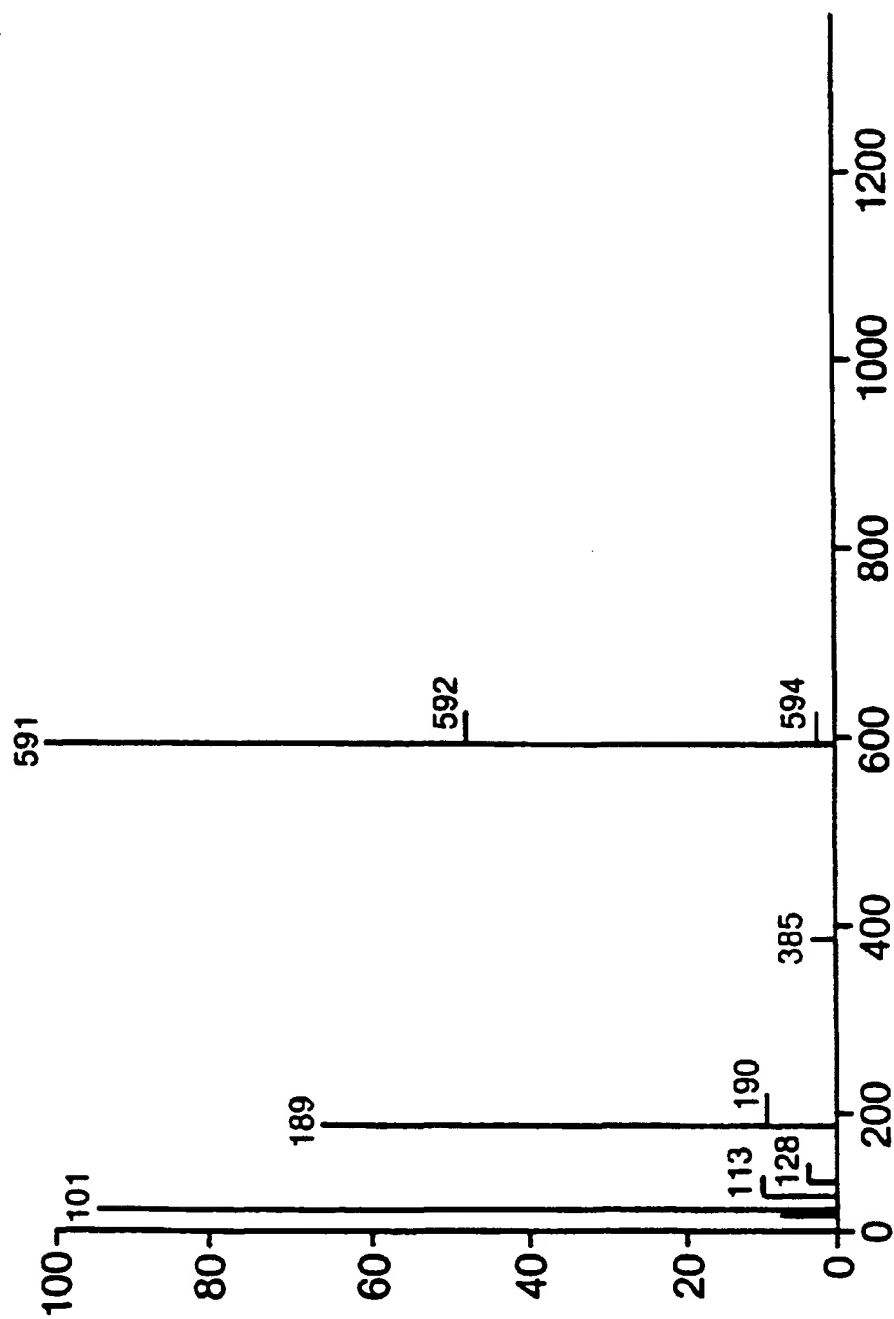


FIG.8

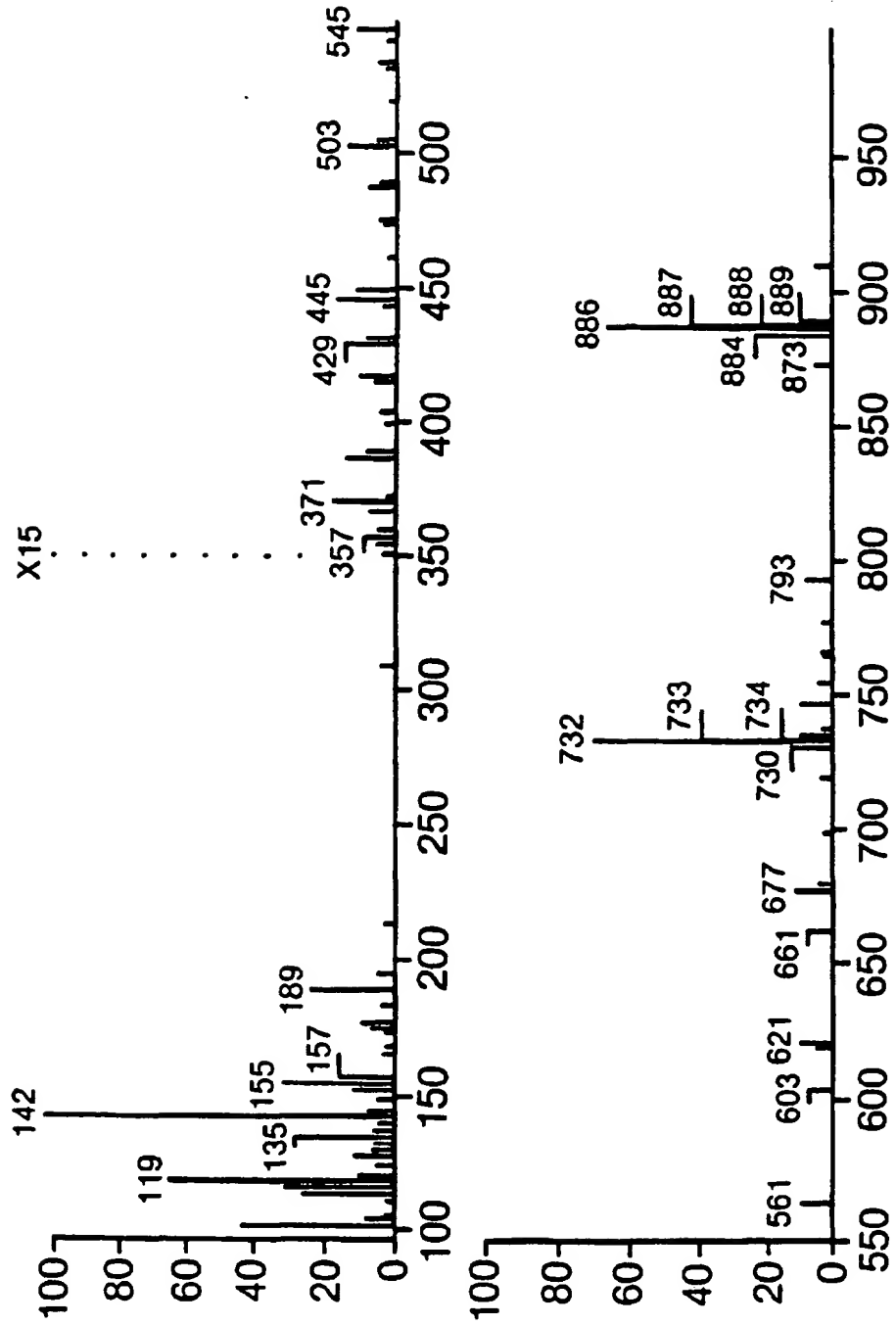


FIG. 9

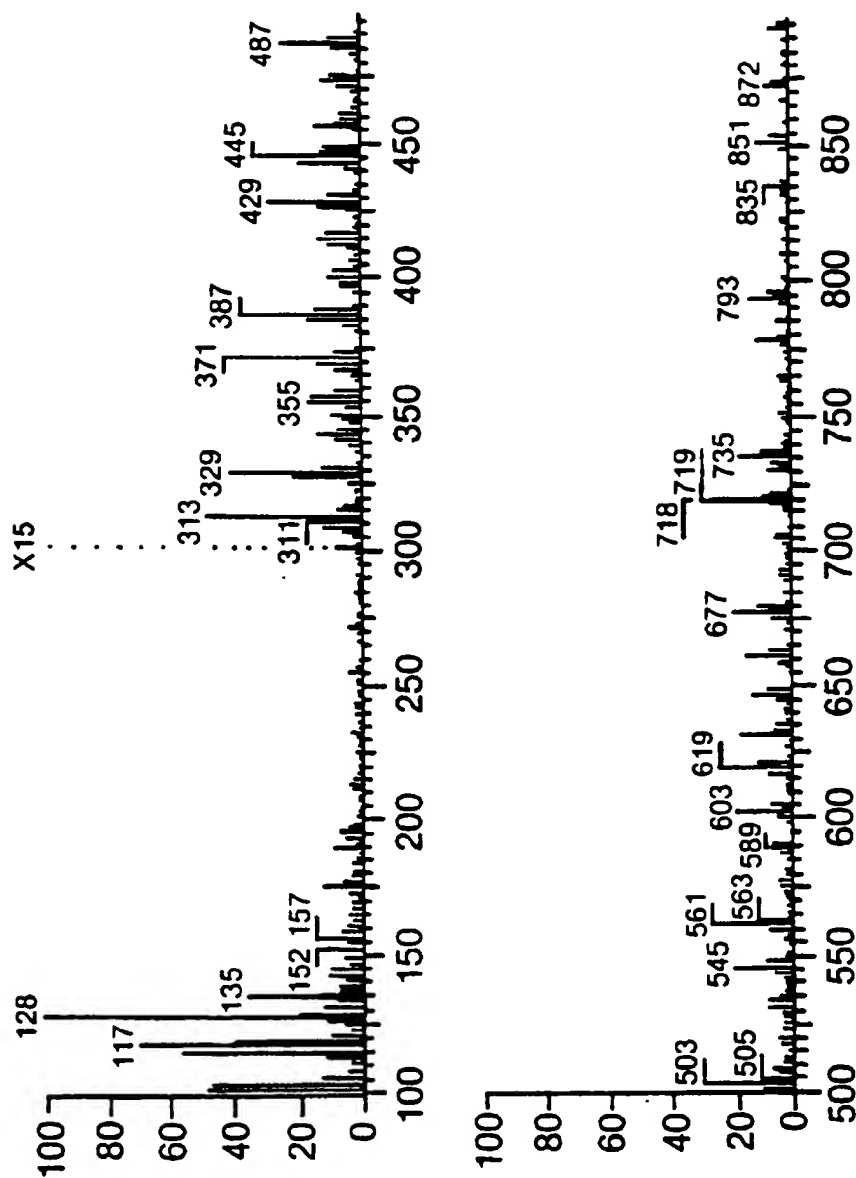
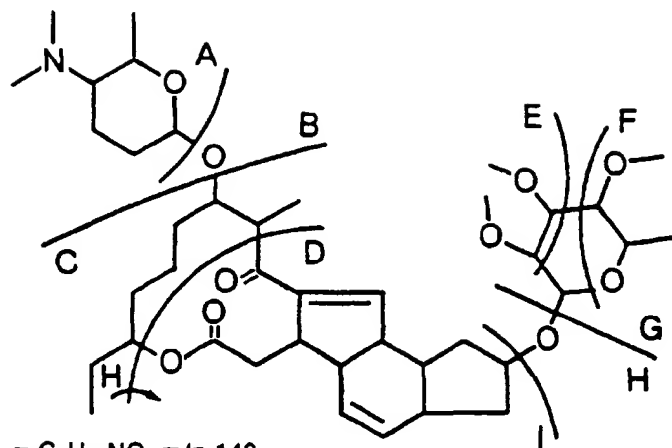


FIG.10



- A = $C_8H_{16}NO$, m/z 142
 B = $C_8H_{16}NO_2$, m/z 158
 C = $C_{33}H_{49}O_8$, m/z 573
 D = $C_{24}H_{33}O_8$, m/z 449
 E = $C_4H_8O_2$, m/z 88
 F = $C_4H_8O_2$, m/z 88
 G = $C_9H_{17}O_4$, m/z 189
 H - $C_8H_{17}NO_2$ = $C_{24}H_{31}O_4$, m/z 383
 I - $C_8H_{17}NO_2$ = $C_{24}H_{31}O_3$, m/z 367

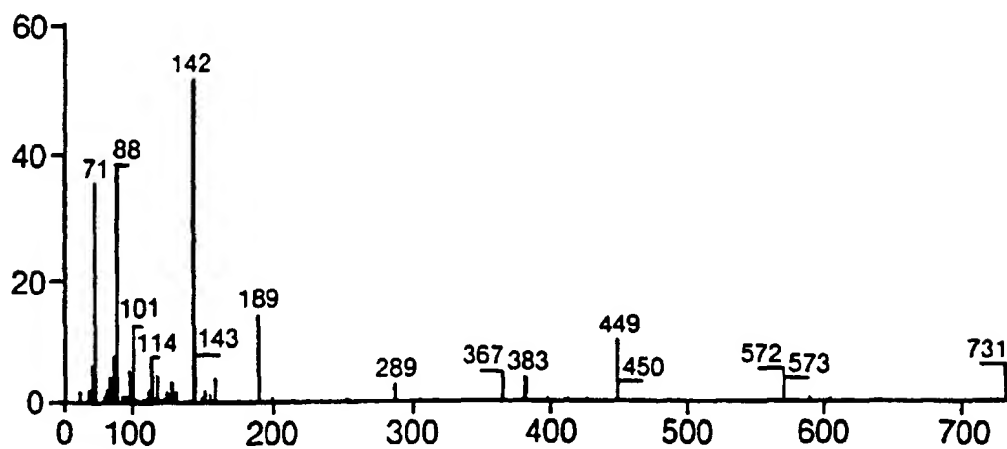


FIG. II

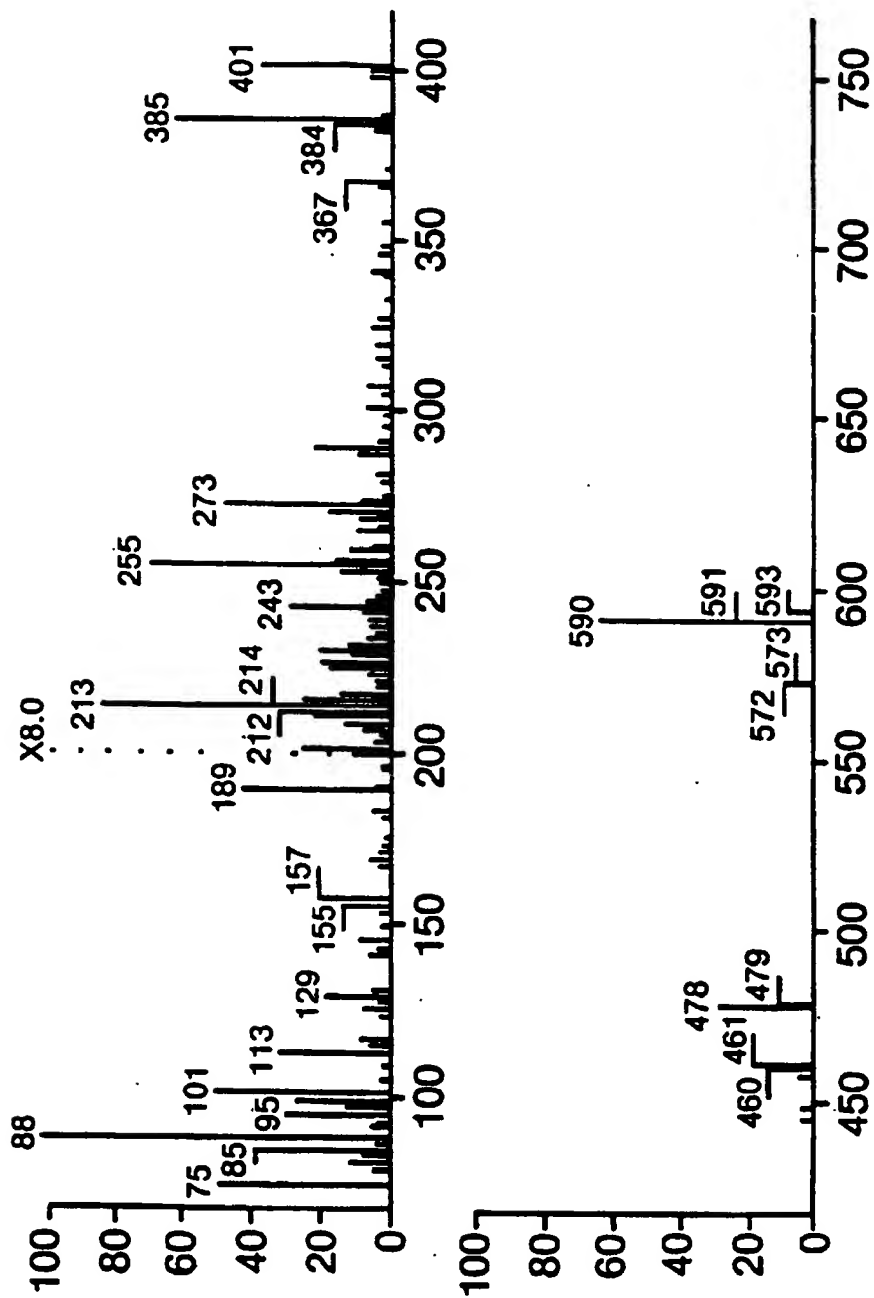


FIG.12

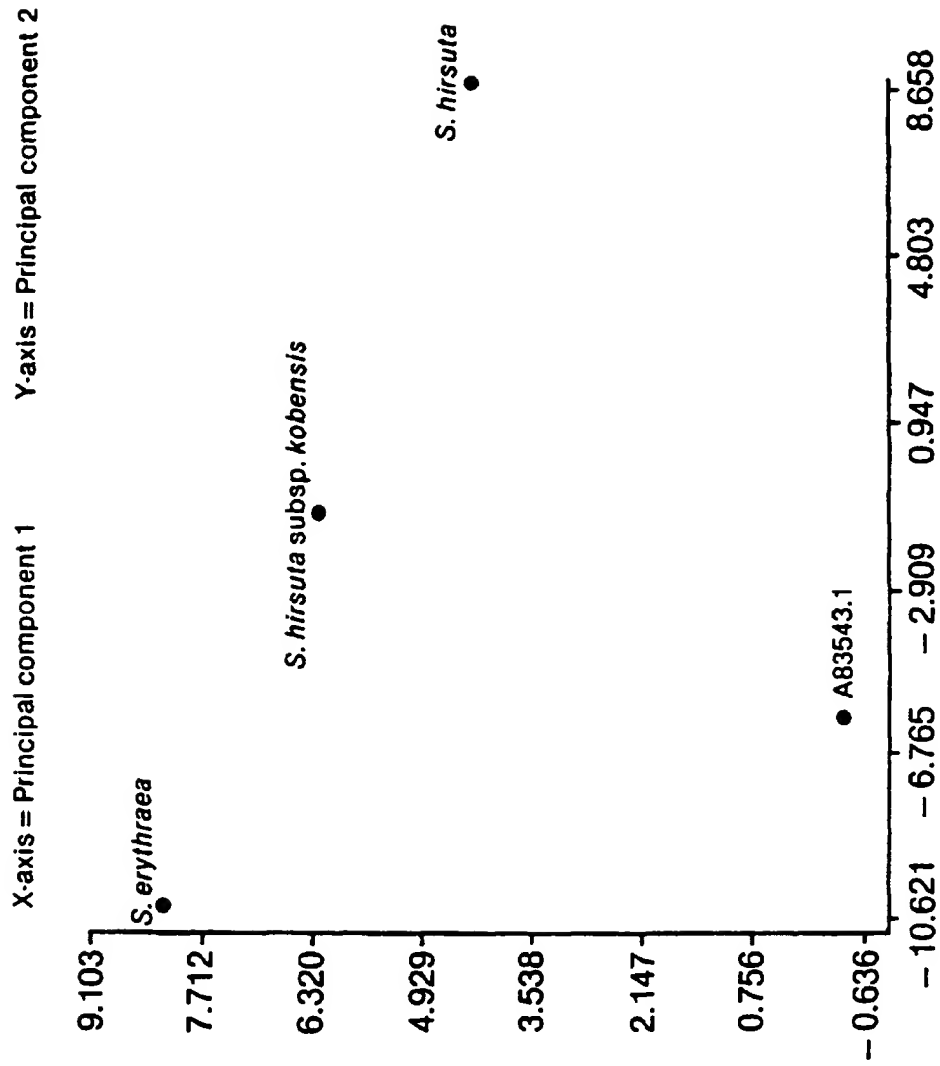


FIG.13

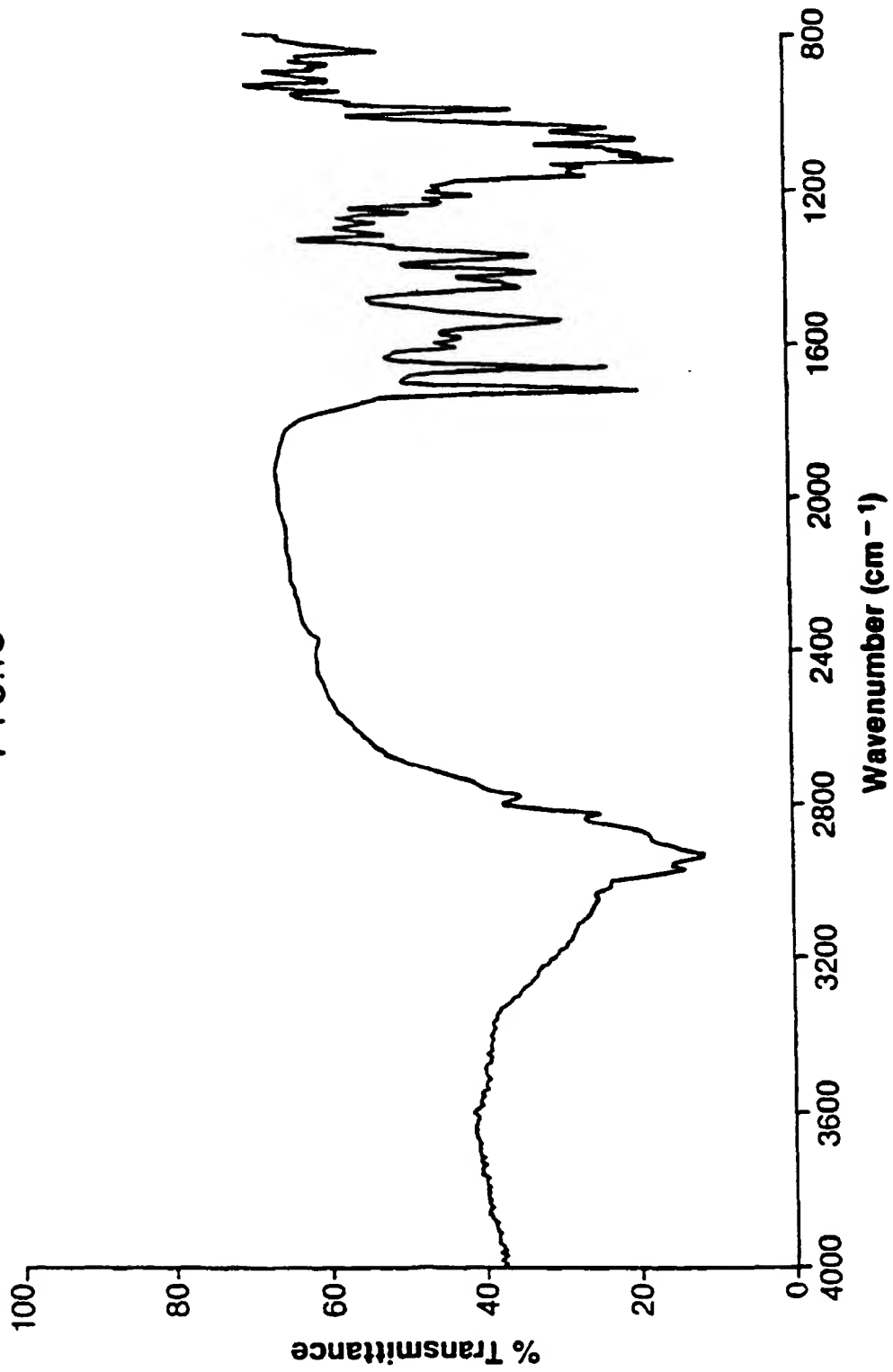


FIG.14

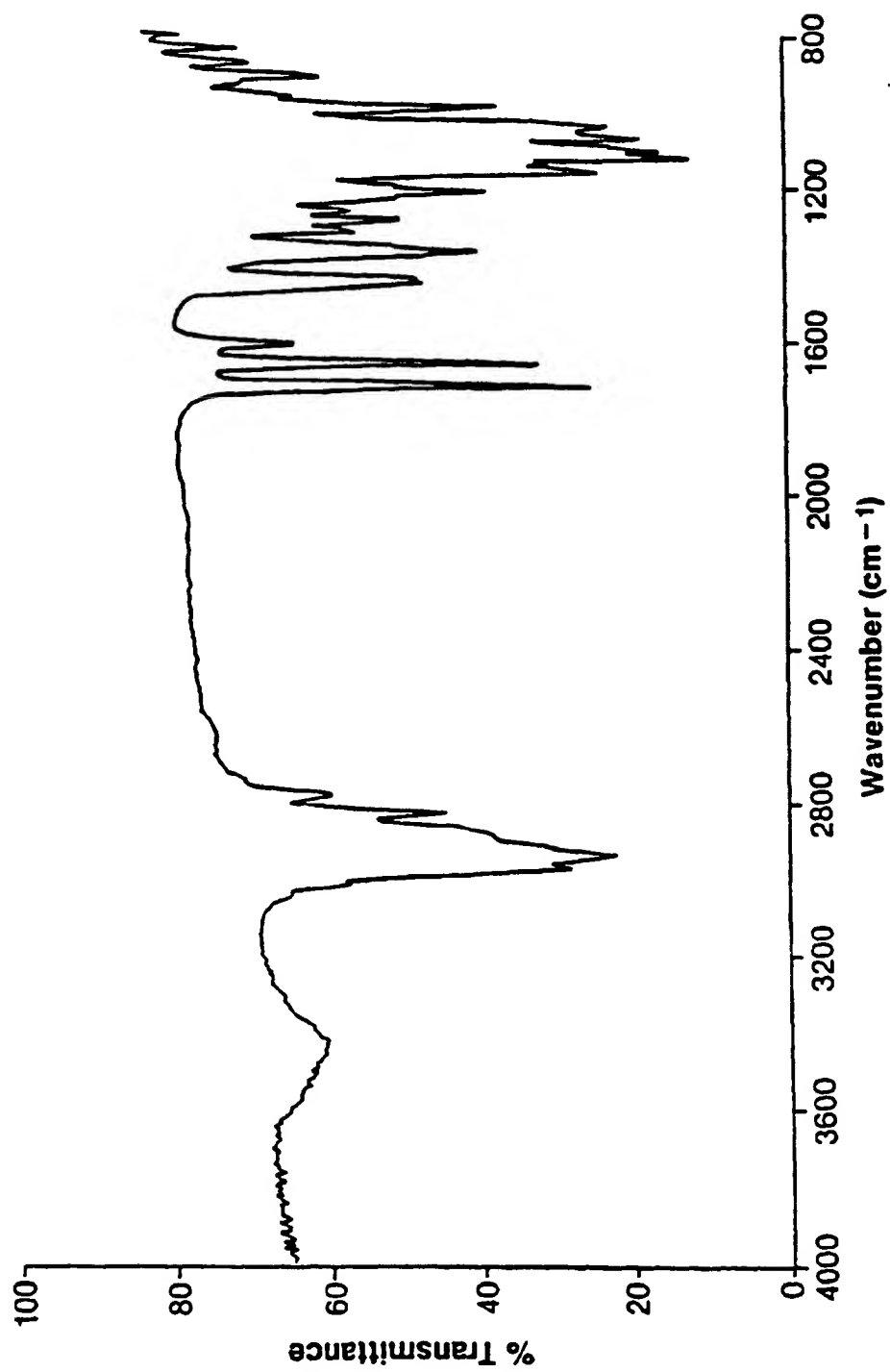


FIG.15

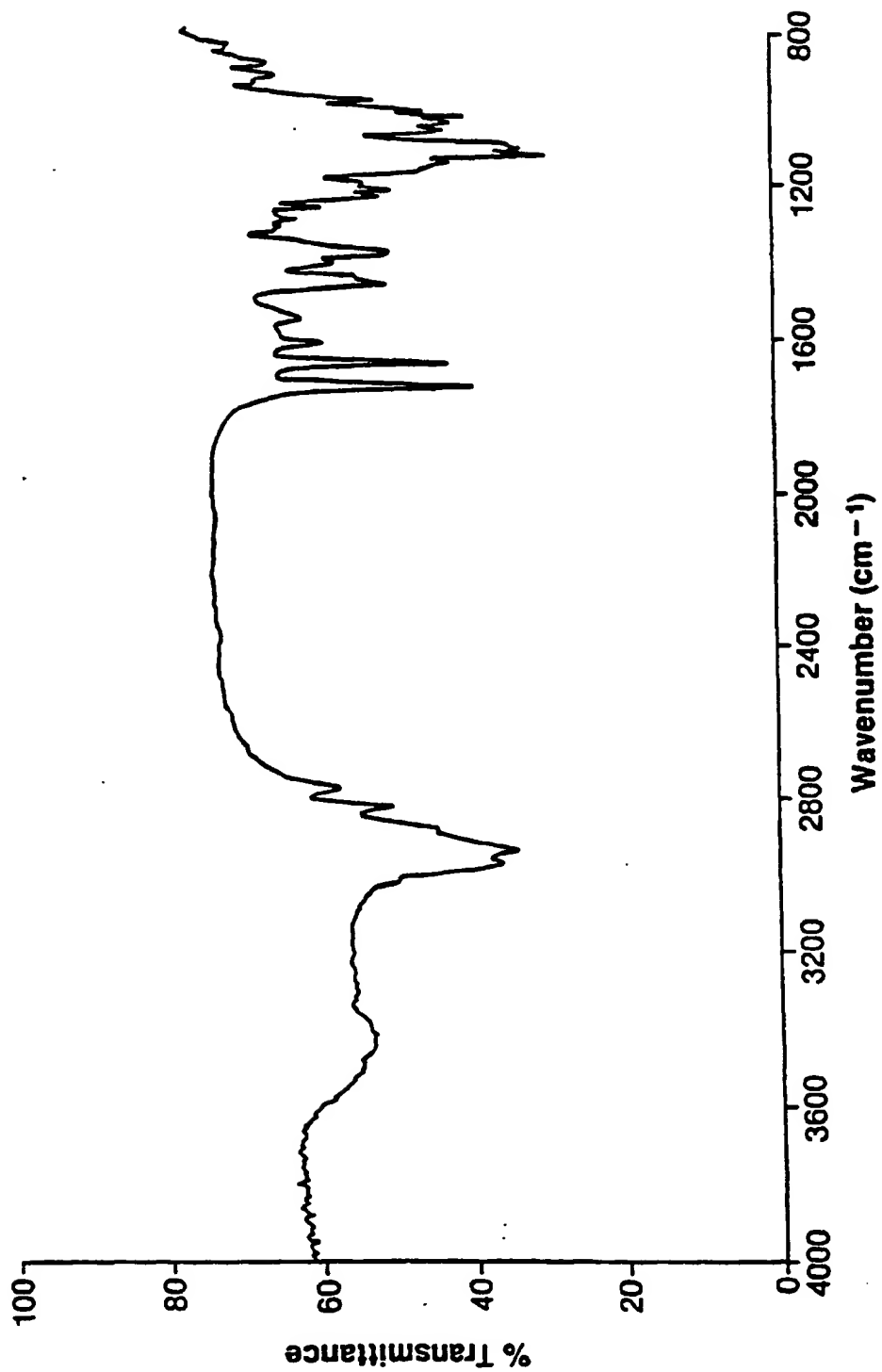


FIG.16

